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Director
Prof. Claudio Pignata

PhD Thesis

NEW APPROACHES IN MANAGEMENT AND TREATMENT OF PRIMARY IMMUNODEFICIENCIES AND OTHER GENETIC DISEASES

Candidate
Dr. Maria Pia Cicalese

Tutor
Prof. Claudio Pignata
Federico II University
Co-Tutor
Prof. Alessandro Aiuti
HSR-TIGET

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GENERAL BACKGROUND, SUMMARY OF RESEARCH ACTIVITY AND AIMS OF THE THESIS

Primary Immunodeficiencies (PIDs) consist of a group of monogenic diseases, all characterized by impaired immune responses of either innate and/or adaptative immunity. They lead to a variety of manifestations, including vulnerability to pathogens and opportunistic microbes, allergy, inflammation, autoimmunity and cancer. The incidence of PIDs varies from 1 in 600 to 1 in 500,000 live newborns, depending upon the specific disorder (1, 2). More than 260 disorders have been identified up to now, deriving from mutations in over 300 different genes, thanks to recent next generation sequencing technologies (3-5).

During my PhD program in "Human Reproduction, Development and Growth" (XXVII Cycle, years 2011-2014), I contributed to the development of new approaches for the treatment of PIDs and other genetic diseases by advanced therapies, such as gene and cellular therapy.

Moreover, I participated in the clinical and biological characterization of patients with immunodeficiencies and in the implementation of new approaches for the clinical management of children affected by hematological and immunological disorders undergoing bone marrow transplantation.

My other lines of research have been focused on the use of new drugs in pediatric population, characterization of severe and unusual conditions and description of the State of the Art of known patologies in childhood.

In my thesis I will describe the main points mentioned above.

Research activity in PIDs is fervid and continues to produce new information on genes involved in differentiation and function of the immune system cells and on new mutations and molecular mechanisms. Gene sequencing has allowed to link different clinical presentations to single gene defects that were thought in the past to display a traditional presentation only. On the same time, this underlines the role of other factors, both genetic and non-genetic, in the regulation of gene expression (6). In the present thesis, I will present two papers I participated to, dealing with clinical and biological characterization of patients affected by PIDs. The first consists in the evaluation of the

intergenerational and intrafamilial phenotypic variability in a cohort of familial cases carrying a 22q11.2 deletion, while the second is related to the analysis of the regulatory and effector T cell functions in a CD25 deficient patient harboring a novel IL2RA mutation.

Noteworthy, some working groups (WG) emerged and are active in studying several aspects of rare and severe primary immunodeficiency diseases worldwide. The Primary Immune Deficiency Treatment Consortium (PIDTC), a network of 33 centers in North America, is an example of working group actually running projects of study and characterization of PIDs. Current protocols address the natural history of patients treated for severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS), and chronic granulomatous disease (CGD) through retrospective, prospective, and cross-sectional studies. Moreover, the establishment of partnerships with European and other International colleagues, the work with patient advocacy groups to promote community awareness and the setting of prospective treatment studies to determine optimal therapies and the future directions of clinical research for PIDs are other major purposes (7).

In Italy, the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) is a good example of network that coordinates the work of many onco-hematological pediatric centers across the nation, carrying out projects of surveys and promoting the drawing up of recommendations, guidelines and diagnostic and therapeutic protocols in pediatric patients affected by haematological and oncological diseases. As a member of this association, in this thesis I will present some works I contributed to, in the context of the AIEOP Supportive Therapy WG, Infectious Diseases WG and Bone Marrow Transplant WG, dealing with new approaches for the clinical management of children affected by hematological and immunological disorders undergoing bone marrow transplantation.

Many PIDs are life-threatening, notably PID affecting T lymphocytes (8) and for this reason hematopoietic allogeneic stem cell transplantation (HSCT) has been used for almost 50 years in the attempt to cure PIDs (9-11). Successful results and improvement of survival and quality of life have been achieved for many PIDs, as severe combined immunodeficiencies (SCIDs); however, for other T cell immunodeficiencies, as Wiskott-Aldrich Syndrome (WAS), hemophagocytic lymphohistiocytosis (HLH), chronic granulomatous diseases (CGDs) and several other diseases, the procedure is still

associated with complications and mortality, especially due to the immune conflict between the donor and the host immune system, leading to rejection and graft-versus-host disease (GvHD) and infectious complications related to myeloablative conditioning regimens. These issues are mostly overcome when a HLA genoidentical donor is available, but the outcome can be poor in other settings, despite the recent progresses in new conditioning regimens, GvHD prevention, immune manipulation and unrelated donors' choice (4, 9-11).

Early diagnosis and treatment remain a main issue for all forms of PID to prevent organ damage and life-threatening infections and to improve prognosis and quality of life (6, 12). New methods for neonatal detection of PID have been recently developed and have been shown to be a suitable screening for the vast majority of severe immunodeficiency diseases characterised by T- or B-lymphopenia in newborns (6, 12-14).

In this scenario, gene therapy (GT) has been a challenging new approach in the last years, particularly directed to patients affected by defined PIDs and lacking a suitable bone marrow donor, as a life-saving alternative (15). Despite the advantages of an autologous and "personalized" procedure, and the impressive clinical results on patients with X-linked SCID, ADA SCID, CGD and WAS, gene therapy treatment has been in some cases counterbalanced by serious complications of insertional oncogenesis, drawing attention to the need of new generation of viral vectors and new approaches of gene correction with superior safety characteristics (15-17). The data regarding the long term follow-up of ADA SCID patients treated with γ-retroviral vector GT in San Raffaele Institute in the last two decades are presented as the main study of this thesis. Moreover, I will present preliminary results on patients treated more recently with LV-based hematopoietic stem cells (HSC) GT in our Institute.

Clinical application of new therapeutic strategies has extended in the last two decades to several other genetic diseases, including blood borne, such as hemoglobinopathies and sickle cell disease (17), and metabolic diseases; among the latter category, relevant results have been achieved with gene therapy in patients with X-linked adrenoleukodystrophy (18) and metachromatic leukodystrophy (MLD) (19). The last mentioned trial has been conducted by LV-based approach in San Raffaele

Institute for Gene Therapy and the publication containing data of the first 3 patients is following presented as a part of my research work.

As one of the most severe forms of muscular dystrophy, though still not permanently curable, Duchenne Muscular Dystrophy (DMD) is currently an object of a number of new therapies entering clinical experimentation or progressing up to phase III studies (20-24). All appear to be safe but, despite early encouraging results, efficacy has still to be reached and, moreover, most are limited to specific subsets of patients. The recent characterization of mesoangioblasts (MABs), a subset of pericytes from mouse, dog and human skeletal muscle that can be expanded in culture and maintain the ability to differentiate into skeletal and smooth muscle (25-27), migrating selectively, when delivered intra-arterially, in downstream inflammed tissues, has made a concrete possibility to perform cellular therapy for murine and canine model of muscular dystrophies (28-30). On the basis of the safety of this approach and partial efficacy in ameliorating the dystrophic phenotype, the same strategy has been attempted in a first-in-human phase I/II clinical trial in DMD children, named "Cell Therapy of Duchenne Muscular Dystrophy by intra-arterial delivery delivery of HLA-identical allogeneic mesoangioblasts", in San Raffaele Scientific Institute, Milan. Having participated in the Study as Scientific Collaborator e Clinical Coordinator, I will show in this thesis the results on the 5 treated patients, that have been recently submitted in a scientific journal.

Chapter 1

“Gene Therapy for Immunodeficiency due to Adenosine Deaminase Deficiency: update on long-term follow-up”

1.1 INTRODUCTION

1.1.1 Adenosine deaminase (ADA) deficiency, an inborn error of purine metabolism

Inborn errors of purine metabolism exhibit broad neurological, immunological, hematological and organ manifestations. These enzymes are widely but not ubiquitously distributed in human tissues and are crucial for synthesis of essential nucleotides. The purine bases (adenine, guanine and hypoxanthine, and the catabolites xanthine and uric acid) are heterocyclic imidazolopyrimidine molecules that are crucial for integral functions in cell physiology. Along with the related pyrimidines, purines form nucleosides and nucleotides that are the vital building blocks for the nucleic acids DNA and RNA, as well as being central to metabolic regulation, energy conservation and transport, formation of coenzymes (the nicotinamide nucleotides, NAD/NADP, and the flavin nucleotides, FMN/FAD), phospholipid and glycolipid synthesis, signal transduction and translation (31, 32), As a result, defects in the metabolic pathways of these important molecules, although rare, can be disabling or deadly (33).

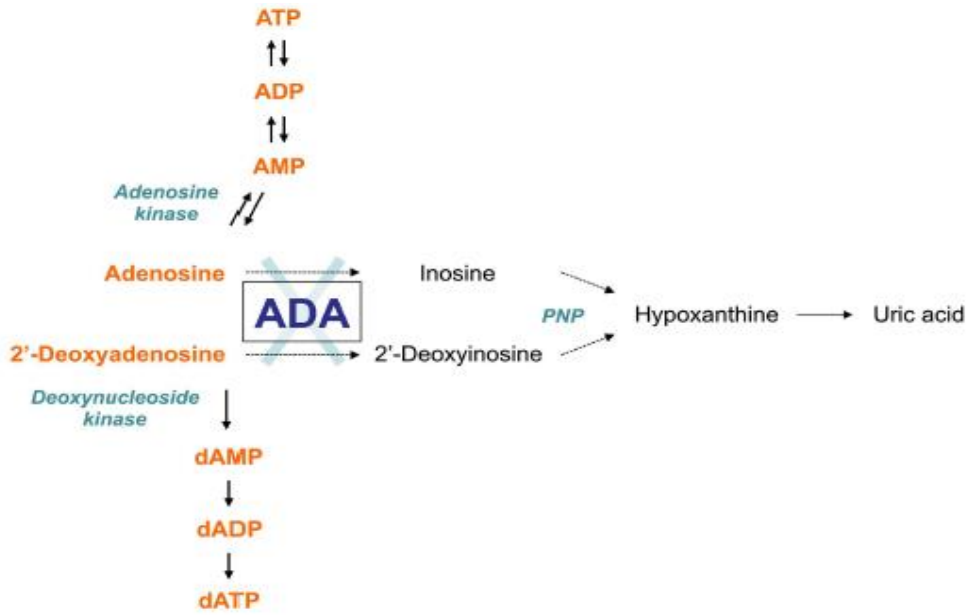
At present, >35 enzyme defects of nucleotide synthesis, salvage and catabolism of both purines and related pyrimidines have been identified (34, 35), but only 17–18 of these are associated with serious clinical consequences (36). These defects are generally rare: the prevalence of these disorders is presently unknown and probably underestimated (33).

As an enzyme of the purine salvage pathway, adenosine deaminase (ADA) catalyzes the deamination of adenosine and 2'- deoxyadenosine, as well as several naturally occurring methylated adenosine compounds (37, 38). The deamination of adenosine and 2'-deoxyadenosine gives rise to inosine and deoxyinosine, respectively (39). Further conversion of these deaminated nucleosides leads to hypoxanthine, which can be either transformed irreversibly into uric acid or salvaged into mononucleosides (33) (**Figure 1.1**). Mutations in the ADA gene cause alterations in ADA activity, enzyme stability, and survival leading to accumulation of Ado, dAdo, and deoxyadenosine nucleotides (dAXP) in plasma, red blood cells (RBCs), and tissues; the concentration of dAXP represents a common measurement of the systemic toxicity level in ADA-deficiency (40). High concentrations of these adenosine-related metabolites

have a profoundly deleterious effect on lymphocyte development through perturbation of multiple intracellular enzymes including ribonucleotide reductase, terminal deoxynucleotidyl transferase, and S-adenosylhomocystine hydrolase. Since the toxic metabolites that accumulate because of the enzyme defect primarily derive from dying cells, it has been hypothesized that each infection results in additional attrition of immune cells and function. In addition to immune cell counts and functionality, intracellular ADA expression levels and purine metabolite concentrations are important biomarkers of ADA deficiency (40).

Although ADA is present in all cell types, its enzyme activity differs considerably among tissues. The highest amounts in humans are found in lymphoid tissues, particularly the thymus, where it plays an important role in lymphocyte development, the brain, and gastrointestinal tract. ADA activity is particularly high in thymocytes of the thymic cortex, but drops off rapidly in the medulla (41). The ADA enzyme is ubiquitously expressed both intracellularly and on the cell surface where it complexes with two molecules of CD26 as a combined protein (42-44).

Figure 1.1: The adenosine deaminase (ADA) metabolism.



Most adenosine derives from endogenous breakdown of ATP and degradation of RNA, or is taken up exogenously by ubiquitously expressed nucleoside transporters. Unlike adenosine, 2'-deoxyadenosine is formed by DNA degradation and is predominantly catabolized by ADA. Further conversion of inosine nucleoside leads to hypoxanthine, which can either enter a non-reversible pathway to uric acid or salvaged back into other mononucleosides. In the absence of ADA, the presence of these alternative "bypass" pathways results in normal concentrations of the catabolic products of the enzyme reaction in patients with ADA-SCID. Conversely, the levels of ADA substrates, adenosine and 2'-deoxyadenosine, are not only found in increased amounts in extracellular body fluids, but they also "spillover" into additional pathways normally only minimally utilized, thus contributing to the pathogenic mechanisms of the disease ***(From Sauer AV et al., Front Immunol 2012).***

1.1.2 Other ADA enzymes disorders

There are two enzymes which carry out ADA activity, named ADA1 and ADA2. ADA1, a 40 kD monomeric protein with a 200 kD, noncatalytic combining protein, is responsible for about 90% of adenosine deamination. ADA2 is larger at 110 kD and appears to play a general adenosine deamination role in serum (43). Relevant disorders arise from deficiencies of ADA enzymes, while rare superactivities of ADA also may occur (33) **(Table 1.1)**.

1.1.2.1 ADA superactivity

An autosomal dominantly inherited Coombs' negative nonspherocytic haemolytic anaemia was reported, with greatly increased erythrocyte ADA activity of approximately 45–to 70-fold elevation (45) **(Table 1.1)**. Milder elevations of erythrocyte ADA activity (two- to six-fold) are found in patients with Diamond-Blackfan anaemia 1 (DBA1; OMIM # 105650), an autosomal dominant disorder with a high degree of clinical and genetic heterogeneity (46). It remains unclear whether ADA has any role in the pathophysiology of DBA, which has been categorised as a ribosomopathy with genetic defects identified in nine different ribosomal genes: RPS19, RPL5, RPS26, RPL11, RPL35A, RPS10, RPS24, RPS7 and RPS17 (47). DBA classical features include normochromic macrocytic anemia, reticulocytopenia and nearly absent erythroid progenitors in the bone marrow.

Patients show growth retardation, and approximately 30 to 50 % have craniofacial, upper limb, heart and urinary system congenital malformations. The majority of patients have increased mean corpuscular volume, elevated erythrocyte ADA activity and persistence of hemoglobin F (33, 48).

1.1.2.2 ADA2 deficiency

CECR1 (cat eye syndrome chromosome region, candidate 1, OMIM # 607575) encodes ADA2, an adenosine deaminase (ADA) isoform that has partial structural homology with human ADA1. Both proteins catalyze the deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively, but the affinity of ADA2 for adenosine is lower than that of ADA1 by a factor of approximately 100 (49) **(Table 1.1)**.

ADA2 is a secreted homodimer highly expressed in plasma, whereas ADA1 is monomeric and largely intracellular (50). Whole-exome sequencing identified loss of-function mutations in CECR1 in three unrelated patients with a syndrome of intermittent fevers, early-onset lacunar strokes and other neurovascular manifestations, livedoid rash, hepatosplenomegaly, systemic vasculopathy and mild immunodeficiency (33).

Subsequent candidate-gene sequencing was performed in three patients with a similar phenotype, as well as two young siblings with polyarteritis nodosa and one patient with small-vessel vasculitis (33).

Skin, liver and brain biopsies revealed vasculopathic changes characterised by compromised endothelial integrity, endothelial cellular activation and inflammation. Immunologic assessment showed mild abnormalities, such as hypogammaglobulinemia and increased B-cell death and reduced B-cell differentiation. Patients had significantly decreased ADA2 activity in plasma, and Western blot analysis showed reduced ADA2 protein levels in cell lysates, consistent with a loss of function. ADA1 specific activity was preserved (33).

Studies in patients' cells and in zebrafish support the hypothesis that ADA2 is a growth factor for endothelial and leukocyte development and differentiation. These data also suggest that ADA2 deficiency may compromise endothelial integrity while polarising macrophage and monocyte subsets toward pro-inflammatory cells, establishing a vicious circle of vasculopathy and inflammation (49, 51, 52). Potential therapeutic strategies may include fresh-frozen plasma or recombinant ADA2, since ADA2 is found in plasma, and bone marrow transplantation, given that monocytes and macrophages, the main producers of ADA2, are derived from bone marrow (33, 49).

Table 1.1 ADA enzymes disorders

Disorder, gene symbol, chromosomal location (OMIM)	Enzyme defect	Clinical features	Diagnosis/metabolic tests	Treatments
Autosomal recessive combined immunodeficiency *ADA*, 20q13.11 (102700)	Deficiency of adenosine deaminase (ADA)	Early onset severe combined immunodeficiency (SCID): in 1st year of life for 80-85% of cases,[29] profound lymphopenia (T/B-cell deficiency), recurrent opportunistic infections, failure to thrive in infancy; some reports of skeletal abnormalities[30], with cupping, flaring of costochondral junction, mild pelvic dysplasia; sensorineural hearing loss reported[31]	Raised erythrocyte deoxy-ATP, high urine/ plasma deoxyadenosine; absent erythrocyte activities of *ADA1* and SAHH (*AHCY*). Deoxy-ATP and SAHH* are used to assess clinical severity and monitor effectiveness of therapy.[32]	Bone marrow transplant (BMT)[34] or haemopoietic stem cell therapy (HSCT). Enzyme replacement therapy with PEG-ADA[35], red cell encapsulated ADA[33]
		Late onset combined immuno-deficiency: clinical immune deterioration[32], diagnosis after 1st year/first decade for 15-20% of cases[32], chronic lung infections, viral infections including warts.[33]	Mildly raised erythrocyte deoxy-ATP, partial/ low erythrocyte *ADA1* and *SAHH** activities.[33]	Gene therapy trialled.[36-38]
Autosomal dominant anaemia, Diamond–Blackfan anaemia (DBA), *gene unknown* (102730	Adenosine deaminase (ADA) 'superactivity'	Coombs' negative nonspherocytic haemolytic anaemia with splenomegaly[39] Congenital aplastic anaemia (Diamond–Blackfan syndrome)[40]	Grossly elevated (45-70x) activity of erythrocyte ADA (inceased transcription), mildly raised plasma urate.[41] Milder elevations of erythrocyte ADA activity (2- to 6-fold). Erythrocyte ADA has a supporting diagnostic value in DBA due to its specificity in distinguishing patients with DBA from non-DBA individuals[41]	Not established
Adenosine deaminase 2; cat eye syndrome, chromosome region candidate 1; *CECR1*, 22q11.1 (607575)	Deficiency of adenosine deaminase (ADA2)[42]	Intermittent fevers, early-onset lacunar strokes and other neurovascular manifestations, livedoid rash, hepatosplenomegaly, systemic vasculopathy and mild immunodeficiency[42]	Hypogammaglobulinemia, decreased IgM, varying degrees of lymphopenia, development of lupus anticoagulant, decreased ADA2 activity in plasma[42]	None established yet, but potential strategies inlcude fresh-frozen plasma, recombinant ADA2 and bone marrow transplantation[42]

Modified from Balasubramaniam S., J Inherit Metab Dis, 2014.

1.1.3 ADA1 deficiency or ADA SCID

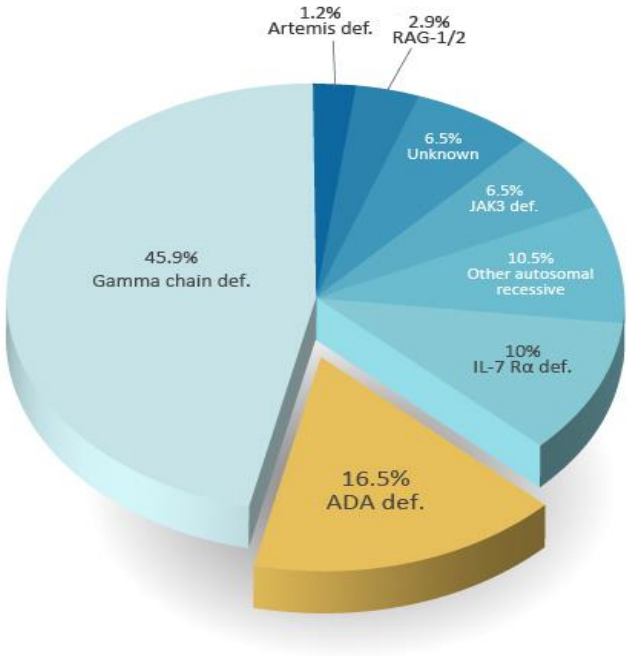
Deficiency of ADA1 (ADA-SCID, OMIM # 102,700) is the second-most prevalent form (approximately 15%) of all SCID cases (53, 54) **(Figure 1.2)** and represents one-third of cases of autosomal recessive SCID (55). ADA-SCID affects every year 1:200,000 to 1:1,000,000 live births, both males and females, with autosomal recessive inheritance. The causative gene is the *ADA* gene, a 12-exon/32 kb gene located on chromosome 20q13.12 (56). The overall incidence in Europe is estimated to range between 1:375,000 and 1:660,000 live births (55).

ADA-SCID was first reported in 1972 in two patients with severely impaired cellular immunity (57). Since then, many advances have been reported in the diagnosis, management and treatment for this disease **(Figure 1.3).** Current available therapeutic options include bone marrow transplantation (BMT), enzyme replacement therapy with bovine ADA (Peg-ADA), or hematopoietic stem cell gene therapy (HSC-GT) (44).

ADA-deficient patients suffer from lymphopenia, severely impaired cellular and humoral immune function, failure to thrive, and a rapidly fatal course due to infection (39). Moreover, autoimmune manifestations are commonly observed in milder forms of the disease (44).

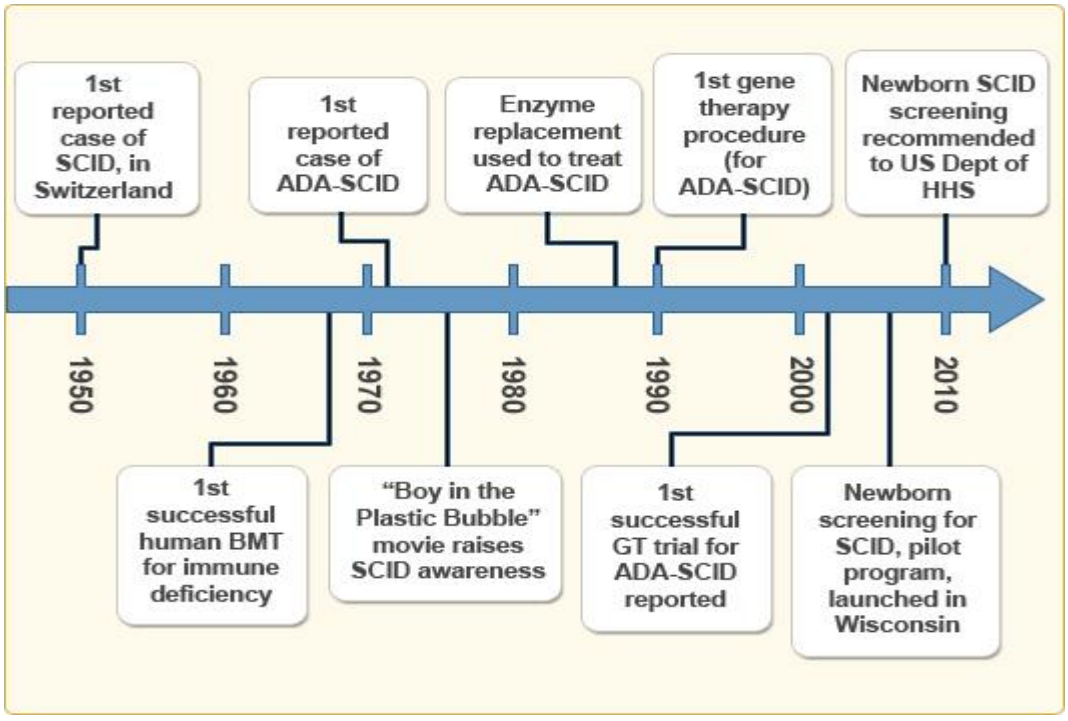
While in rare cases SCID may be diagnosed at birth, the average age at diagnosis is approximately 4 months (58). In any case, an early diagnosis and treatment is mandatory to prevent ADA SCID symptoms and also to get the best therapeutic effect, because the chances for treatment success are highest for those who have not yet experienced severe opportunistic infections (59). For this reason, diagnostic methods to make early diagnosis are important to significantly improve patient health outcomes, while at the same time reducing the cost of medical care.

Figure 1.2 Relative frequency of the different molecular defects in SCID.



From Buckley RH. Annu Rev Immunol. 2004;22:625–655.

Figure 1.3 Timeline of Advances in SCID.



1.1.3.1 Genotype/Phenotype Correlations

ADA is the only gene to cause ADA SCID (60). Sequence analysis can identify most known *ADA* pathogenic variants, except for large deletions, which are detected by deletion/duplication analysis. To date, more than 70 different mutations in the ADA gene have been identified, distributed as follows: 60% substitution mutations, 20% splicing mutations, 13% deletion mutations and 7% termination mutations (nonsense) (61).

ADA deficiency is associated with a broad clinical and mutational spectrum. Defining the relationship of genotype to phenotype among patients with different degrees of immunodeficiency has been complicated because the disease is rare, most mutations are 'private' and patients are often heteroallelic. In recent years, knowledge of ADA structure and systematic expression of mutant alleles have revealed that phenotype is strongly associated with the sum of ADA activity provided by both alleles (62).

The clinical expression of immunodeficiency can be influenced strongly by environmental factors, such as exposure to and treatment of pathogens, and also by genes (other than that for ADA) that affect responses to infection (56). Somatic mosaicism due to *in vivo* reversion to normal of an inherited mutation in the *ADA* gene has also been observed in some cases of *ADA* SCID (60, 63, 64). Nevertheless, at the extremes, phenotype and residual ADA activity clearly are related: blood mononuclear cells from healthy subjects with partial deficiency have 5%–70% of normal ADA activity, versus <1%–2% in cells from patients with SCID. From reports of new ADA mutations, some splicing and missense alleles appear to be associated with delayed and late-onset phenotypes (60, 65-69). This suggests that allele combinations providing more than some critical level of functional mutant enzyme (or of normal ADA from splicing mutants) (66, 70) can confer a milder phenotype than SCID.

Arredondo Vega et al. (56) noticed that expressed ADA activity of wild-type and mutant alleles ranged over five orders of magnitude. The disease-associated alleles expressed 0.001%–0.6% of wild-type activity, versus 5%–28% for alleles from "partials". A relationship with erythrocyte deoxyadenosine nucleotide (dAXP) levels was also established (**Table 1.2**).

A scale for ranking novel *ADA* alleles based on expression may have utility for newborn screening for primary immunodeficiency disorders (62).

Table 1.2 Total Expressed ADA Activity and Erythrocyte dAXP levels for Phenotype Groups.

Phenotype	No. of Patients	RBC dAXP (Range) [nmol/ml][a]	Expressed ADA Activity (Range) [nmol/hr/mg protein][a]
SCID	23	766 ± 344 (273–1,839)[b]	67 ± 96 (1–314)[b]
Delayed onset	8	186 ± 55 (100–263)	308 ± 149 (72–522)
Late onset	3	122 ± 104 (28–234)	855 ± 122 (714–926)
Partial	3	15 ±12 (3–27)	29,144 ± 39,247 (927–73,963)
Normal	...	<2	525,184 ± 43,180

[a] Mean ± SD.
[b] $P < .0001$, SCID vs. delayed onset.

From Arredondo-Vega. Am J Hum Genet. 1998.

1.1.3.2 Clinical characteristics and their pathogenesis

Immune defects

The majority of patients (80–85 %) show an early onset severe form of ADA SCID with absence of cellular and humoral immunity and hypoplasia or apparent absence of lymphoid tissue (59, 71), which present within 1 to 6 months of age with multiple recurrent infections by fungal, viral, and opportunistic agents and a rapidly fatal course (57, 72).

Lymphopenia and attrition of immune function over time are the two findings common to all presentations of ADA deficiency. Recent studies have shed additional light on the mechanisms leading to abnormal T-cell development and function. Analysis of the thymus of an ADA-deficient patient revealed severe atrophy as well as loss of cortex-medulla demarcation (73). Immature thymus epithelial cells were abundant while Autoimmune Regulator expressing mature thymus epithelial cells were absent. Dendritic cells were severely depleted while macrophage-like cells were increased, possibly because of the need to remove apoptotic thymocytes. Another group investigated causes for the impaired responses of ADA deficient T cells to stimulation.

CD4+ T cells from ADA-deficient patients had severely compromised TCR/CD28-driven activation, including hypo-phosphorylation of Zeta Associated Protein-70, Ca2+ flux and ERK1/2 signaling, resulting in impaired proliferation and cytokine production (74). Hence, diverse mechanisms might account for abnormal function of T cells in ADA deficiency. This leads to severe depletion of all three major categories of lymphocytes, T-, B-, and NK-cells (72, 75, 76).

Total immunoglobulin levels may be only slightly depressed at birth due to the maternal contribution of IgG, whereas both IgM and IgA, which ordinarily do not cross the placental barrier, are often absent. However, once IgG levels decline as maternal antibodies are cleared, a pronounced hypogammaglobulinemia signals the absence of humoral immunity (39, 77).

ADA SCID onset occurs between 6 months and the first few years in 10-15% of patients with a "delayed" clinical manifestation and from second to fourth decades in a smaller percentage of patients with "late" onset, showing less severe infections and a slowly deteriorating immune function (59, 71).

Finally, the benign condition called "partial" ADA deficiency is characterized by a decreased enzyme activity in erytrocytes, but a residual enzyme activity in leukocytes and other nucleated cells (70).

Delayed or late-onset patients have significant immunodeficiency, but variable clinical manifestations (69). In some cases, residual ADA activity (approximately 5% of normal) supports generation of normal numbers of lymphocytes (71). These delayed or late onset forms show progressive immunological and clinical deterioration, often associated with autoimmune manifestations, including hemolytic anemia, and immune thrombocytopenia (78, 79). Serum immunoglobulin levels are altered in late-onset patients, with IgG2 levels being highly reduced or absent. IgE levels are elevated and often associated to eczema and asthma. An inability to produce antibodies against polysaccharide and pneumococcal antigens was frequently found in ADASCID patients with milder forms of the disease (80, 44).

Non-immune defects

The initial and most devastating presentation of ADA-SCID is due to the immune defects (81). Nonetheless, several non-immune abnormalities have been described in ADA deficiency, indicating that this disease should be considered a

systemic metabolic disorder (39, 79) and more complex than other forms of SCID (75, 82). ADA is ubiquitously expressed in all cell types; when absent, the systemic metabolic toxicity is frequently associated with organ damage (83). These include failure to thrive (75, 76); hepatic and renal disease (82, 84); skeletal alterations such as cupping and flaring of the costochondral rib junctions; brain, lung and bone marrow alterations that will be discussed in detail below for their relevance.

Complications from infections usually predominate in the clinical presentation of infants with ADA deficiency, therefore, the full spectrum of non-immunologic manifestations and their natural course may be obscured (85). Several abnormalities have been described in low numbers of patient, and might reflect effects of infectious agents rather than primary defects due to ADA deficiency: i.e., renal and adrenal abnormalities, phyloric stenosis, and hepatic disease (39, 44).

- *Brain abnormalities*

ADA-deficient patients may suffer neurological deficits, developmental delay and sensory-neuralhearing loss secondary to infections, autoimmunity or transplantation (85-87). Additionally, neurological abnormalities, such as spasticity, hypotonia, head lag, nystagmus, difficulties in focusing gaze, seizures and inability to focus and high frequency sensorineural deafness have been reported (85, 88, 89), which were not explained by infections or medications (90). Computed tomographic scans and cranial MRI of these patients revealed volume loss as well as basal ganglia and thalamus abnormalities, possibly due to accelerated nerve cell death or altered stimulation of adenosine receptors (71). Cognitive and neurobehavioural abnormalities as below-average IQ, hyperactivity, attention deficits, among ADA-deficient infants have been also described, commonly seen also in other inborn errors of purine metabolism, suggesting that abnormal purine homeostasis might be directly responsible for their development (71).

- *Lung abnormalities*

Many ADA-deficient patients suffer from severe lung disease, which in a few cases has been attributed to infections and/or immune dysregulation. Yet, in most ADA-deficient patients no respiratory pathogens are isolated (91).

Lung abnormalities were recently reviewed among 16 ADA-deficient patients (92).

Broncho-alveolar lavage and lung biopsies in affected patierns identified accumulation of Periodic Acid Schiff positive, surfactant-like granular material as well as macrophages engulfing lamellar bodies, typical of pulmonary alveolar proteinosis (PAP) in seven patients (43.8%). To confirm that ADA deficiency causes PAP and to better understand the pathogenesis of PAP, the same group showed that ADA-/- mice suffer from many of the metabolic, immune and systemic features observed in ADA-deficient patients and develop lethal respiratory disease by 3 weeks of age, due to accumulation of surfactant in the alveoli (93). Importantly, early restoration of ADA activity in ADA-/- mice prevented the development of PAP, thereby confirming that ADA deficiency directly contributes to PAP formation (71).

- *Bone Marrow abnormalities*

Neutropenia in ADA-deficient patients has often been related to autoimmunity or infections (94). Recently hypocellular bone marrow and mild dysplastic changes of three hematopoietic lineages were also identified in an ADA-deficient patient (95). Moreover, 13 ADA-deficient patients exhibited various abnormalities in their peripheral neutrophils including prolonged neutropenia, hypogranular and vacuolated neutrophils as well as hyperlobular and pyknotic neutrophils (96). These patients also had large/giant platelets, as well as atypical eosinophils with cytoplasmic vacuoles, uneven granulation and hyperlobular nuclei. Bone marrow examinations performed in a few ADA-deficient patients revealed myelodysplastic changes. Importantly, there was an inverse relationship between phosphorylated ADA substrates and neutrophil numbers suggesting a primary myelotoxic process (96). Studies in ADA-/- mice provided additional insight into the potential effects of impaired ADA function on the bone marrow (82).

Fibroblast colony formation assays showed that ADA-deficient bone marrow mesenchymal progenitor cells grew normally, but these cells supported long-term culture initiating colony formation less efficiently than wild-type cells. Proteome profiling of total cytokine and chemokine production from the supernatant of the cultures revealed reduced levels of M-CSF, IL-6, CXCL1/10, and soluble Intra-Cellular Adhesion Molecule 1. Importantly, the percentage of early hematopoietic stem cells was significantly lower in the bone marrow of ADA_/_ mice (82), although it is still not clear whether this reflects a primary hematopoietic defect or is secondary to a stroma defect (71).

1.1.4 Diagnosis of ADA SCID

A suspicion of ADA SCID should be considered an emergent condition in an infant with a serious infection, as its early detection dramatically improve the chances of survival (72, 97).

Physical examination reveals absence of lymphoid tissue and the thymus is radiographically undetectable. The presence of skeletal abnormalities may aid in the diagnosis (98).

Immunologic evaluation must be accomplished as quickly as possible. Characteristic laboratory abnormalities include severe, age-adjusted lymphopenia and pan-hypogammaglobulinemia – only present after loss of maternally inherited immunoglobulins -, reduced or absent major lymphocyte subpopulations (T, B and NK cells) by flow cytometry analyses and absent or profoundly reduced T-cell proliferation to mitogens and antigens (72).

Maternal T-cells may engraft in some patients with ADA SCID and obscure the peripheral blood lymphocyte phenotype. On occasion, engrafted maternal T-cells may become activated by HLA disparities and cause clinical graft-vs-host disease, to be suspected in infants with diffuse cutaneous eruptions and/or other clinical and laboratory suspicion of ADA SCID and to be distinguished from erythrodermia associated with Omenn Syndrome (97).

The assessment of ADA catalytic activity is useful for the confirmation of ADA SCID diagnosis. Affected individuals who have not been transfused have less than 1% of normal ADA catalytic activity in erythrocyte hemolysates or in DBS extracts, while affected individuals who have been recently transfused may require testing of fibroblasts or leukocytes (99). Analysis of ADA enzymatic activity in plasma is not useful for diagnosis because ADA activity is much lower in plasma than in cells, even in non ADA-deficient individuals, and because plasma contains adenosine deaminase activity caused by ADA2. ADA2 has a much lower affinity for substrate and, unlike ADA1, is insensitive to inhibition by EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine), which allows ADA1 and ADA2 to be quantitated independently. ADA activity measurement sometimes can give misleading results: a severe defect in ADA activity can be found in clinically unaffected subjects with a ‘partial’ ADA deficiency, because

variable residual ADA activity expressed in cells different from immune cells can be sufficient to maintain good immune function (59).

Measurement of metabolites is absolutely mandatory to confirm the diagnosis of immunodeficiency due to ADA deficiency (59, 99). These markers help to confirm the diagnosis and to monitor therapies intended to restore ADA function:

- Elevated deoxyadenosine triphosphate (dATP) or total dAdo nucleotides (dAXP) in erythrocytes:Mature red cells normally lack dAXP. In the absence of ADA, excessive dAdo phosphorylation leads to a marked increase in dATP and total dAXP, a finding pathognomonic for ADA deficiency (it does not occur with ADA2 deficiency). In untreated affected individuals, the level of dAXP in hemolysates or DBS extracts correlates with clinical phenotype (SCID > "delayed/late" onset > "partial ADA deficiency"). Even after transfusion, some elevation in erythrocyte dAXP persists and strongly indicates underlying ADA deficiency.
- Reduced S-adenosylhomocysteine hydrolase (AdoHcyase, SAHase) activity in erythrocytes: Owing to inactivation by dAdo, erythrocyte AdoHcyase activity is less than 5% of normal.
- Elevated Urinary dAdo: Excretion of dAdo is markedly elevated in untreated affected individuals.
- Elevated levels of Ado and dAdo in extracts of dried blood spots (DBS), quantified using tandem mass spectrometry, is a sensitive method for screening neonates for ADA deficiency (14, 100 101).

Sequence analysis of the *ADA* gene is also available to confirm diagnosis.

1.1.5 Newborn screening

There is a consensus that nearly all cases of SCID could be diagnosed at birth if routine blood counts were done and flow cytometry, as well as T-cell counts, were performed when lymphocyte levels are below the normal range in newborns. Once a diagnosis is confirmed, treatment can begin immediately (102, 103).

The profound reduction in T lymphocytes in nearly all forms of SCID can be identified in DBS from neonates by demonstrating a reduced level of T-cell receptor excision circles (TRECs) in extracted DNA. TREC screening is now performed in several states in the US * and may be adopted by others. Universal newborn screening has helped to establish the true incidence of SCID in California (1 in 66,250 live births) and has led to the improvement of survival outcome (6). The potential use of TRECs evaluation from DNA of DBS as method to evaluate ADA SCID has been proposed but, unfortunately, this approach is expensive, it takes a long time and it lacks of sensitivity in diagnosis of late and delayed onset ADA SCID (59).

As about 15% of all cases of SCID result from ADA deficiency, biochemical testing for ADA deficiency should be performed as a follow up in all newborns who are positive by TREC screening. Reduced levels of a B lymphocyte marker, kappa-deleting recombination excision circles (KRECs), have also been found in DNA from DBS of individuals with delayed- or late-onset ADA deficiency (100).

ADA SCID screening by tandem mass spectrometry for analysis of metabolites from DBS could potentially be an easier and cheaper method (13).

* The States and US Territories screening all newborns for SCID as of August 2014 are: California, Colorado, Connecticut, Delaware, Florida, Illinois, Iowa, Maine, Massachusetts, Michigan, Minnesota, Mississippi, New Jersey, New York, Ohio, Oregon, Pennsylvania, Rhode Island, Texas, Utah, Washington, West Virginia, Wisconsin, Wyoming, District of Columbia and Navajo. Louisiana and Puerto Rico have both stopped screening due to a lack of funding. Ontario, Canada is also screening all newborns for SCID (104).

1.1.6 Autoimmunity in ADA SCID

Immune dysregulation involving B cells, with multiple forms of autoimmunity, has been described in ADA SCID patients (44).

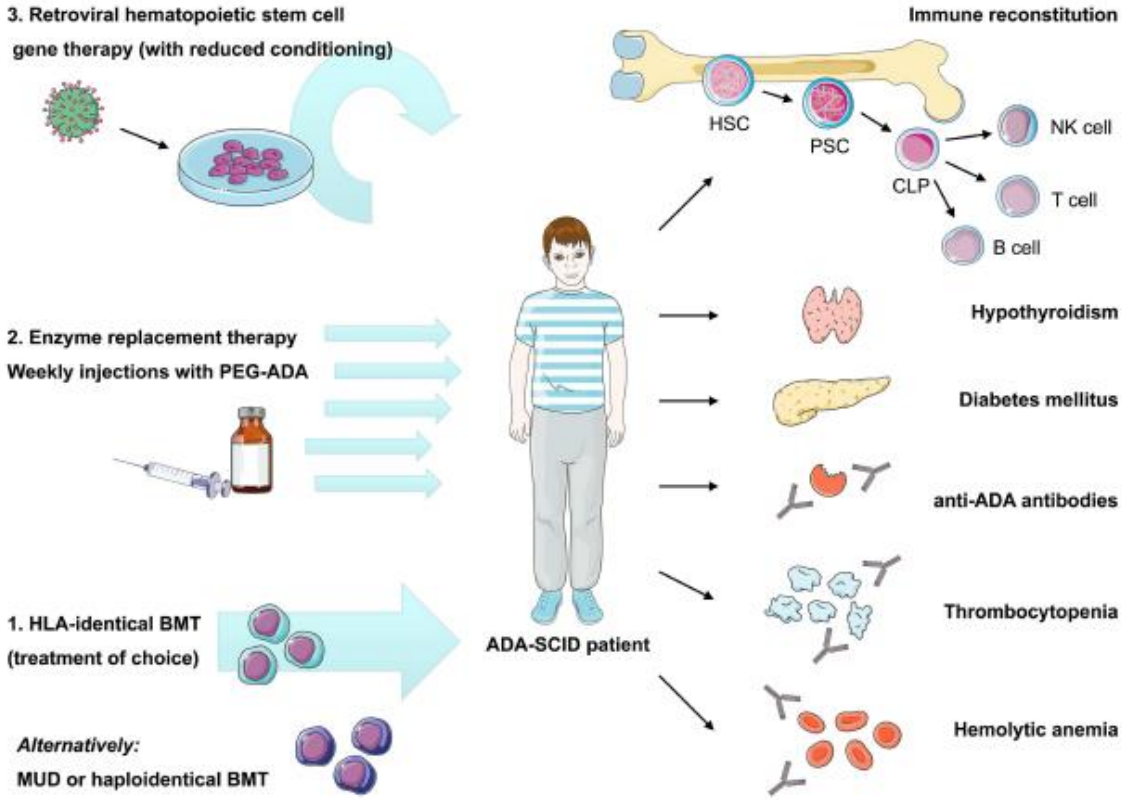
Autoimmune dysregulation is frequently observed in patients with milder forms of the disease or late-onset patients. They may manifest as autoimmune hypothyroidism, diabetes mellitus, hemolytic anemia, and immune thrombocytopenia (69, 105) **(Figure 1.4**). Similar complications, such as autoimmune hemolytic anemia and autoimmune thyroiditis, have also been reported in patients after long-term PEG-ADA treatment (38, 69, 81, 105, 106). No specific reports on immune dysregulation or autoimmunity in BMT-treated ADA-SCID patients are available in literature (106). Nevertheless, autoimmune manifestations have been reported in larger single-center studies on BMT-treated patients with various kinds of immunodeficiencies, including ADA deficiency (107, 108). The major immune dysregulations observed in both studies included thyroid autoimmunity, autoimmune hemolytic anemia, and glomerulonephritis (107, 108). Autoimmune manifestations have also been described in patients treated with HSC-GT (109). Four ADA-SCID patients, including one patient that already showed immune dysregulation while on PEG-ADA, developed signs of autoimmunity, such as hemolytic anemia, thrombocytopenia, autoimmune hepatitis, and autoimmune thyroiditis (109 and unpublished observation).

Since varying degrees of immune reconstitution can be achieved by the available treatment options for ADA-SCID, break-down of tolerance and development of autoimmunity can represent a major concern.

Two studies assessed defects in the lymphoid compartments of ADA-SCID patients following PEG-ADA. Different degrees of abnormalities in the B-cell compartment and inability to respond to vaccines, despite the presence of normal serum-Ig or hypogammaglobulinemia were reported (110). Moreover, a retrospective longitudinal analysis in ADA-SCID patients treated with PEG-ADA showed that decreased levels of newly produced B cells underlie the progressive and significant decrease in circulating B cells in these patients (106). Since long-term PEG-ADA treatment is associated with abnormalities in B cell subsets, but often also with a decrease in T-cell functions (110), a limited B or T-cell repertoire combined with alterations in peripheral tolerance could further favor breakdown of tolerance (**Figure 1.5**).

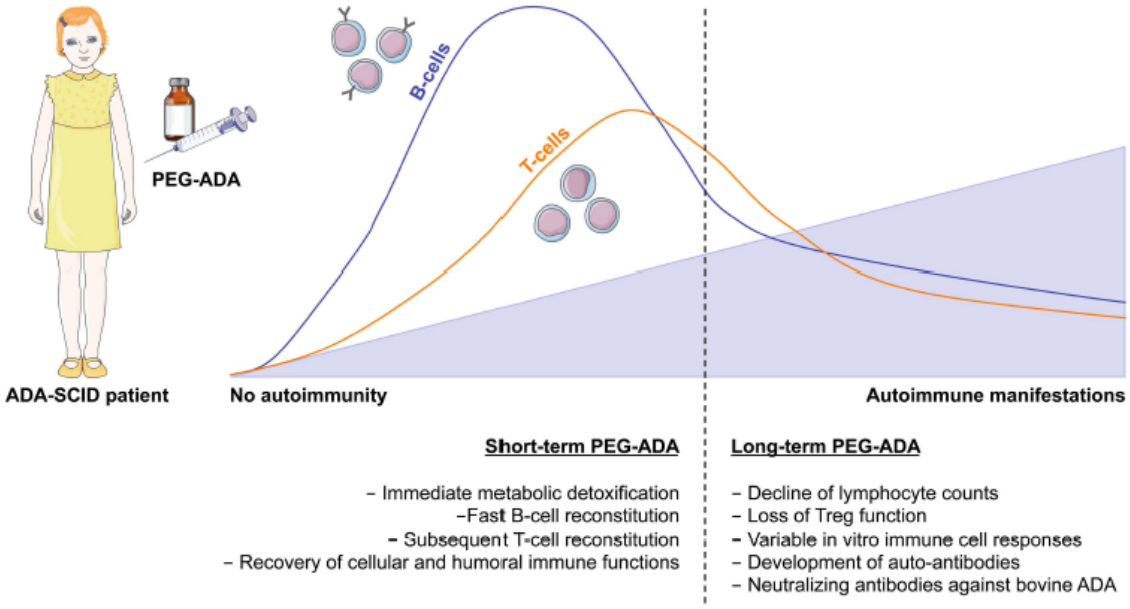
A recent study (111) also demonstrated a significantly reduced frequency of CD4+CD25+FoxP3+ regulatory T cells among ADA-deficient patients and that these cells had reduced ability to suppress proliferation of co-cultured autologous responder T cells. Similarly, regulatory T cells from ADA-deficient mice had significantly reduced suppressive activity. Whether regulatory T cell abnormalities are also responsible for Omenn's syndrome that has been described in ADA-deficient patients (112) is still not clear. The autoimmunity observed in ADA-SCID might also be due to defective central and peripheral B cell tolerance. Indeed, new emigrant/transitional and mature naive B cells from ADA-SCID patients were shown to contain excess auto-reactive clones. Moreover, B cell receptor and Toll-like receptor functions in B cells are impaired after ADA inhibition, which might also contribute to B-cell tolerance defects (113).

Figure 1.4 Current therapeutic options in ADA-SCID and reported autoimmune manifestations after treatment.



Immune reconstitution in ADA deficiency can be achieved by bone marrow transplantation, enzyme replacement, or gene therapy, nonetheless recovery of immune functions may vary depending on the applied treatment and patient's characteristics. Treatment of choice remains bone marrow transplantation from a HLA-identical sibling donor, while transplants from alternative donors are associated with high morbidity and mortality. Enzyme replacement therapy using pegylated bovine ADA is a non-curative treatment requiring weekly intramuscular injections with PEG-ADA. ADA-SCID has been the pioneer disease for the development of human gene therapy. It is based on the reinfusion of autologous HSC transduced with a retroviral vector containing the *ADA* cDNA. Variable degrees of immune reconstitution can be achieved by these treatments, but onset of autoimmunity is of concern in post-treatment ADA-SCID patients. Reported autoimmune manifestations include: autoimmune hypothyroidism, diabetes mellitus, thrombocytopenia, hemolytic anemia, and development of anti-ADA antibodies ***(From Sauer AV et al., Front Immunol 2012).***

Figure 1.5 Immune reconstitution and development of autoimmunity after PEG-ADA treatment.



Enzyme replacement therapy with pegylated bovine ADA is a lifesaving but non-curative treatment for ADA-SCID patients. It provides metabolic detoxification and protective immune function with patients remaining clinically well, but immune reconstitution is often suboptimal and may not be long-lived. Shortly after initiation of PEG-ADA treatment, patients show recovery of B-cell counts, followed by a gradual increase in T-cell numbers and reconstitution of immune cell functions. However, the long-term consequences of PEG-ADA treatment are unknown. Immune recovery in B and T- cells is below normal levels. Major concerns are the susceptibility to opportunistic infections and the development of autoimmunity due to lymphopenia with gradual decline of immune functions and perturbation of T- and B-cell tolerance ***(From Sauer AV et al., Front Immunol 2012).***

1.1.7 Therapies for ADA Deficiency

Bone marrow transplantation with allogeneic HSC has long been considered the mainstay of ADA-SCID treatment. However, unlike other forms of SCID, ADA-SCID patients can be treated with pegylated bovine ADA (PEG-ADA), which it not approved in the EU but is frequently available under compassionate use, or in clinical trials of Autolotous HSC-GT (109, 114). The availability of different treatment modalities presents an opportunity for improved patient care but also difficulties in deciding upon the specific choice of treatment for individual patients (**Figure 1.4**). Making the correct choice is further complicated by the fact that ADA deficiency is not purely an immune defect, and that the systemic manifestations, which can be of major clinical consequence, must also be managed (81).

1.1.7.1 Hematopoietic stem cell-transplantation

Hematopoietic stem cell-transplantation (BMT) from allogeneic human leukocyte antigen (HLA)-compatible sibling donors resulting in long-term survival and effective immune reconstitution is the treatment of choice for patients with ADA-SCID and other severe variants of primary immunodeficiencies. Since less than 20% of ADA-SCID patients have access to HLA-matched family donors, transplants are often performed from mismatched family or matched unrelated donors (81, 115, 116).

In a series of 475 patients with SCID from the European SCETIDE database, 51 patients with ADA-SCID were included, and the study documented a 3-year survival of 81% for HLA-matched transplantations and 29% for HLA-mismatched transplantations with no information on the degree of immune recovery (115). Only 4 unrelated donor transplantations were reported in this series, and no outcome data on these patients were presented. A subsequent report on 699 patients with SCID included 75 patients with ADA-SCID, but the specific outcome for the patients with ADA-SCID alone was not documented (9). A series of 15 patients with ADA-SCID showed overall survival (OS) of 80%, but there were only 6 transplantations from mismatched family donors and 2 transplantations from unrelated donors in this series (85).

A recent retrospective analysis on the specific outcome of transplants for ADA-SCID, performed by *Hassan et al.* in 2012, collected data from several multicenter studies and analyzed the survival of 106 patients who received a total of 119 transplants

in the past 30 years (58), representing to date the only definitive analytical study of a large number of ADA-SCID children given HCT. BMT from matched sibling (MSD) and family donors (MFD) had a significantly better overall survival (86 and 83%) in comparison to BMT from matched unrelated (MUD) (67%) and haploidentical donors (HAPLO) (43%), while the outcome in mis-matched unrelated donor (MMUD) recipients was unacceptable (29%). Comparison of OS in MSD HCT with MMUD, HAPLO and MUD HCT showed a significant difference ($P < .05$). These data indicate that despite recent progress in transplantation, the use of alternative donors is still associated with a reduced overall survival (58, 81). This is further complicated by the fact that ADA-SCID patients are more difficult to transplant especially from unrelated and haploidentical donors possibly due to their need for conditioning and the underlying metabolic nature of the disease (81, 82).

The large *Hassan* study also addressed the controversial question for preconditioning of SCID children before HCT. A significantly improved OS was observed in unconditioned transplantations (78%) in comparison with transplantations after MAC (78% *vs* 56%; $P = .009$), while transplantations after RIC had an OS of 67%. Indeed, almost all unconditioned transplants had the benefit of HLA matching, so the superior survival was not unexpected, and ADA-deficient cells may be more susceptible to toxic effects of preconditioning agents (118). Despite the lack of conditioning in most MSD/MFD transplantations, a high rate of engraftment was observed (40 transplantations in the 2 groups with an engraftment rate of 90%), while non-engraftment was a major problem after unconditioned haploidentical transplants (58).

When the type of conditioning within transplantations from different donor sources was evaluated, it was shown a good survival in MSD/MFD transplantations even in RIC and MAC regimens, whereas survival was decreased in MUD transplantations and was much worse in MMUD and HAPLO HCT. The degree of HLA disparity appeared to play the major role in determining outcome, irrespective of conditioning regimen (58).

From the analysis of other variables, no difference in survival outcome was found between patients who had received > 3 months PEG-ADA for metabolic detoxification before transplant and patients who had received no or short-term (< 3 months) ERT. A trend to better survival, even if not statistically significant, was observed in babies transplanted < 6 months of age; certainly, a prolonged pretransplant period in an unprotected environment would favour acquisition of a potentially fatal virus or bacterial infection and would contribute to metabolic and post-infectious organ

sequelae. No significant difference in TRM or OS was observed between patients with a more severe phenotype in terms of residual ADA activity and levels of toxic metabolites before transplantation. The source of stem cell used (BM, PBSCs, or UCB stem cells) also had no significant effect on TRM or OS (58).

Long-term immune recovery showed that regardless of transplant type, overall T-cell numbers were similar. Humoral immunity and donor B cell engraftment was achieved in nearly all evaluable surviving patients and most patients were able to discontinue immunoglobulin replacement (58).

According to the longest F-U available data for some patients, the immunological and metabolic recovery after transplant was well maintained even after 10 years or longer (81). Nevertheless, delayed or suboptimal immune reconstitution as a result of poor early engraftment or gradual decline in immune functions was observed in a significant fraction of surviving patients (81).

Infections and graft-versus-host disease (GvHD) still represent major issues in allogeneic transplant setting. In the *Hassan* cohort the majority of deaths (63%) after all types of transplantations were in the first 100 days after HCT, with severe infections (pneumonitis/respiratory failure and sepsis) which formed > 50% of all deaths followed by GVHD and fungal infections. Transplant from MMUD and HAPLO was associated to a higher risk of complications (58). Autoimmune and inflammatory manifestations, persistent infections, and disease-related issues have been described in ADA SCID patients after BMT (85, 88, 107).

The results obtained with transplantation from HLA-identical siblings or family donors indicate that there is a strong correlation between superior donor/host compatibility and outcome both in terms of survival and sustained immune recovery. If a fully matched sibling or family donor is available, the evidence currently available suggests that a transplant should be undertaken without any conditioning. The success rates associated with such procedures and the effective long-term reconstitution in both T- and B-cell compartments does not at present warrant the use of alternative therapies. Some have argued that the use of a conditioning regimen may improve stem cell engraftment and promote an improved quality of immune reconstitution long term. However, the current data do not show any waning of immune function even more than 10 years after unconditioned HSCT, and the majority of patients have remained free of immunoglobulin therapy. Further strong evidence to the contrary would be required

before this recommendation is changed. On the other side, the current evidence suggests that haploidentical donor transplants (performed with or without conditioning) have a poor chance of success and are therefore only undertaken if no other treatment options are available (44, 81, 119).

1.1.7.2 Enzyme replacement therapy with PEG-ADA

Enzyme replacement therapy (ERT) with PEG-ADA (Adagen®) was developed as life saving, not curative treatment for patients lacking a HLA-compatible donor, when risks associated with a partially mismatched transplant do exist, or when graft failure is high as in the delayed- or late-onset phenotypes. PEG-ADA has also been used as a secondary therapy in patients who have failed to engraft following an unconditioned BMT/SCT, or in whom an acceptable recovery of immune function has not been achieved following experimental gene therapy (81).

PEG-ADA is not approved in any EU Member State and it is available through compassionate use or special laws in the EU. It requires life-long weekly or bi-weekly i.m. injections and regular monitoring of plasma ADA activity and red blood dATP. Recent published results on patients who received PEG-ADA between 1986 and 2008 showed that the overall probability of 20 year-survival on PEG-ADA is estimated to be 78% (81).

Attachment of PEG through lysine residues confers several therapeutically beneficial properties to ADA (120, 121). This chemical modification of the bovine enzyme reduces its immunogenicity and prevents its degradation by plasmatic proteases as well as the binding of neutralizing antibodies (120, 121). Thereby the circulating life of the compound is prolonged from minutes to days as clearance from the circulation is inhibited (122). Cellular uptake of PEG-ADA is insignificant and its distribution is limited to the plasma. Enzymatically active ADA continuously circulates and eliminates accumulating adenosine and 2'-deoxyadenosine metabolites (123). The principle of exogenous PEG-ADA administration is based on the direct conversion of accumulating ADA substrates in the plasma and the indirect reduction of intracellular toxic metabolites by diffusion (44).

To date more than 150 patients worldwide have received this treatment (81, 122). In general, PEG-ADA treatment seems to be well tolerated, with clinical benefits

appreciable after the first month of therapy (**Figure 1.5**). Studies have shown that upon the initiation of PEG-ADA therapy, the absolute numbers of circulating T- and B-lymphocytes and NK-cells increase and protective immune function develops (124). Although only limited information is available, some analysis indicated that about half of PEG-ADA treated patients discontinued IVIg (81), whereas long-term follow-up suggests that immune recovery is often incomplete (122). Two retrospective studies showed that despite initial improvements, the lymphocyte counts of all PEG-ADA treated patients were below the normal range at all times. A gradual decline of mitogenic proliferative responses occurred after a few years of treatment and normal antigenic responses occurred less than expected (106, 125). No toxic or hypersensitivity reactions have been reported with PEG-ADA administration. However, several other side effects have been reported including manifestations of immune dysregulations as autoimmunity (type I diabetes, hypothyroidism, immune thrombocytopenia, hemolytic anemia) and allergic manifestations (69, 105) (**Figure 1.5**). An additional concern with PEG-ADA beyond about 8–10 years is the emergence of serious complications, including lymphoid and hepatic malignancies, and progression of chronic pulmonary insufficiency (81). The main side effect associated with the use of PEG-ADA is the development of anti-ADA antibody. The development of specific IgG antibody to bovine peptide epitopes of PEG-ADA has been reported by several groups and often coincides with an improvement in humoral immunity (126-128). In about 10% of treated patients, inhibitory antibodies lead to the enhanced clearance of PEG-ADA with subsequent decline in metabolic parameters and immune function and therefore requiring increase in dosage, administration of corticosteroids, or cessation of therapy (53, 126, 127).

The high cost of lifelong therapy is another major issue of ERT (81).

1.1.7.3 Gene Therapy

HSCT and ERT are effective therapies, but both have their own limitations. Moreover, some ADA SCID patients may not be candidates for cytoablation due to infectious damage to the lung or liver, or may have a milder phenotype that does not justify the risks associated with allogeneic BMT.

A gene therapy approach for ADA-SCID was developed because it was easier to treat by genetic correction than some other indications. This provided an alternative mode of treatment for patients without a suitable HSCT donor and access to PEG-ADA.

Haematopoietic stem cell gene therapy consists of isolating hematopoietic stem cells from the bone marrow of the patient, transducing them with a recombinant viral vector (such as a retrovirus) encoding a functional copy of the ADA gene that is capable of integrating its genetic material into that of the host cell, and then re-infusing the modified cells into the patient. As it is effectively an autologous transplant, full cytoreductive regimes are unnecessary in SCID diseases and their associated risks are avoided. Because ADA is an enzyme and is not involved in processes controlling cell proliferation/apoptosis, tight gene expression regulation has not to date been shown to be crucial and overexpression is unlikely to cause significant side effects (129).

Gene corrected cells are expected to have a selective advantage over their deficient counterparts in a toxic environment, similar to what is seen in clinical cases of somatic mosaicism, where patients experience immune recovery after the emergence of a clone that has reverted the mutation in the ADA gene to normal (*Hirschhorn R. In vivo reversion to normal of inherited mutations in humans. J Med Genet 2003; 40(10): 721-8. Liu P, Santisteban I, Burroughs LM, Ochs HD, Torgerson TR, Hershfield MS, et al. Immunologic reconstitution during PEGADA therapy in an unusual mosaic ADA deficient patient. Clin Immunol 2009; 130(2): 162-74*). Patients may still experience clinical benefit even if ADA normal levels are not attained since as reported elsewhere, individuals expressing as low as 5% of the normal ADA activity can have normal immune functions (131, 132).

1.1.8 Gene Therapy Clinical Studies for ADA SCID

The first attempts at developing a gene therapy (GT) protocol to treat ADA-SCID patients were published in the 90's (**Table 1.3**) (133, 134). The strategy of these studies was, initially, to obtain lymphocytes from the patient blood and modify them with ADA-coding retroviral vectors (RV). Although some improvement in antigen-dependent responses, isohemagglutinin titres and lymphocyte ADA levels were observed after gene therapy, the clinical benefit of the treatment could not be clearly evaluated as PEG-ADA was administered alongside the gene modified T-cells. Nevertheless, the trials showed that gene modified cells were able to persist in the circulation for as long as ten years after treatment.

In a second group of studies, it was attempted to infuse gene modified peripheral blood lymphocytes and/or bone marrow progenitors into patients (**Table 1.3**) (135-138). Again, the outcome of the trials was confounded by the simultaneous administration of PEG-ADA. However, it was demonstrated that gene-modified stem cells were able to engraft and give rise to a progeny of marked cells in the periphery.

A number of amendments were introduced into the GT protocols in order to improve the transduction efficiency and engraftment of modified stem cells, as well as to provide a selective pressure for the corrected cells that would ultimately translate into clinical benefit for the patients. The *ex-vivo* culture of human HSCs was refined with cytokine cocktails that stimulated proliferation and maintained the "stemness" of the CD34+ population (139); whereas the retroviral vector transduction efficiency was enhanced by colocalizing the virus and cells onto fibronectin fragments (138, 140).

The engraftment of infused stem cells was optimized by the inclusion of a mild pre-conditioning regimen, to make space for the corrected progenitors (141). Finally, the selective pressure for outgrowth of gene-modified progeny was provided by the withdrawal of ERT prior to GT. This last alteration was justified by the observations of Aiuti *et al.* in the study published in 2002. A 10-fold increase in peripheral gene-marked lymphocytes was observed in a patient in whom ERT had to be suspended due to complications (142).

A third series of trials started in 2000 with these improved conditions. Following 2 pilot studies (141), a phase I-II clinical trial of gene therapy of HSC was initiated at HSR-TIGET in Milan, Italy, in 2002 up to date (**Table 1.4**). Replication-deficient, recombinant retroviruses derived from the backbone of Moloney murine leukemia virus (MLV) were selected for these trials because of the available long-term experience and their ability to efficiently insert the therapeutic gene into the genome of dividing hematopoietic cells. The patients were pre-conditioned with a reduced dose of busulfan and PEG-ADA administration was stopped prior to GT. A report on the results of the first 10 patients was published in 2009 (109). In summary, at 1 year post-transplant, vector-marked cells were found in bone marrow in the CD34+ population (5%), granulocyte (3.5%), megakaryocyte (8.9%), erythroid (3.8%) and B cell progenitor (8%) population. In the peripheral blood, the marking of T, B and NK cells was between 50 and 90%, meaning that in a toxic metabolic environment there is a selective advantage for corrected lymphocytes. ADA levels in blood mononuclear cells and

erythrocytes did not reach the levels observed in normal individuals (33.6% and 1.9% of normal, respectively), but levels were sufficient to observe a substantial decrease of toxic metabolites in the red cell lineage and allow functional immune recovery. There was an increase in the number of circulating naïve T cells and presence of T cell receptor excision circles (TRECs) in CD3+ cells, suggesting that normal thymic activity was restored and importantly, polyclonal T cell receptor repertoires were detected in the patients. The circulating T cells displayed normal proliferative responses to mitogens, alloantigens, Tetanus toxoid and *Candida albicans in vitro*. The number of B and NK cells also increased over time, although, similar to the T cell compartment, less than half of the patients reached normal cell counts. However, the recovered cells exhibited normal functions. Normal serum immunoglobulin levels were detected in five out of ten patients, allowing for discontinuation of immunoglobulin therapy. In these same individuals, antibodies against viral and bacterial antigens were detected after immunization. Most patients were able to lead a normal life after treatment; only two of them had to be restarted on ERT. Importantly, no adverse events related to the therapy have been reported so far.

In 2003 a similar study was initiated in UK by *Gaspar* group on patients who had no matched donor available and who were failing on PEG-ADA. Similar to the Italian trial, ERT was discontinued one month before gene therapy, and the patients were preconditioned with one dose of melphalan instead of busulphan. The results on the first patient were published in 2006, two years after initiation of treatment (138, 143). Lymphocyte counts increased in this patient, alongside a decrease in erythrocyte dATP levels. The recovery of thymic function was confirmed by the detection of TRECs in the CD4+ and CD8+ subsets, and the newly generated T cells showed proliferative responses upon PHA and CD3 stimulation, as well as a normal heterogeneous T cell repertoire. Gene marking was higher in the lymphocyte population (50% in T and NK) than in the myeloid population (less than 0.1%).

More patients have been treated to date (144, and *unpublished data*). Similar to the initial report in patient 1, two additional patients have experienced good immune recovery and metabolic detoxification, and in a fourth, gene-modified cells make dominant contributions to immune recovery even though PEG-ADA was restarted. Failure of gene therapy has been observed in two individuals due to a poor stem cell harvest in the first, and low transduction efficiency in the second. Three patients have been able to stop Ig replacement and of these two have made normal vaccine responses.

Gene Therapy has been safe and well tolerated, although integrations near proto-oncogenes have been detected in the Italian and UK studies (145. and *unpublished data*).

Another clinical trial was conducted at the National Institute of Health and Children's Hospital of Los Angeles, and the results confirm the importance of nonmyeloablative pretransplantation conditioning to achieve therapeutic benefits with gene therapy for ADA SCID patients (146). Ten patients with ADA SCID were treated with GT using 2 slightly different retroviral vectors for the transduction of patients' bone marrow CD34+ cells. Four subjects were treated without pretransplantation cytoreduction and remained on ADA enzyme-replacement therapy (ERT) throughout the procedure, in the assumption that ERT usage could promote gene-modified cell proliferation (147). Only transient (months), low-level (< 0.01%) gene marking was observed in PBMCs of 2 older subjects (15 and 20 years of age), whereas some gene marking of PBMC has persisted for the past 9 years in 2 younger subjects (4 and 6 years). Six additional subjects were treated using the same gene transfer protocol, but after withdrawal of ERT and administration of low-dose busulfan (65-90 mg/m2). Three of these remain well, off ERT (5, 4, and 3 years postprocedure), with gene marking in PBMC of 1%-10%, and ADA enzyme expression in PBMC near or in the normal range. Two subjects were restarted on ERT because of poor gene marking and immune recovery, and one had a subsequent allogeneic hematopoietic stem cell transplantation.

Recently, based on major safety issues arising in clinical trials of retroviral GT for the treatment of SCID-X1, CGD and WAS, that will be discussed below, alternatively strategies based on ADA encoding lentiviruses (LVV), with a safer design and profile than γ-RV, were developed and studied in preclinical ADA SCID models. *Mortellaro et al* developed a SIN LVV in which the expression of the human ADA gene was driven by a PGK promoter. ADA-deficient mice transplanted with ADA-/- bone marrow cells transduced with the ADA encoding LVV were rescued from lethality and corrected for both their metabolic and immunologic defects in a way that was similar to bone marrow transplantation (148). To improve the efficacy of ADA gene transfer in HSC, the group of Dr. Kohn and Dr. Gaspar designed an LVV that included a codon-optimized human cADA gene under the control of the short form elongation factor-1α promoter (LV EFS ADA) that displayed high-efficiency gene transfer and sufficient

ADA expression to rescue ADA -/- mice from their lethal phenotype with good T- and B-cells reconstitution. Moreover, *in vitro* immortalization assays demonstrated that LV EFS ADA had significantly less transformation potential compared to gRV vectors, without clonal skewing (149).

On this basis, a phase I/II trial with the use of LV EFS ADA is actually running in collaboration in UK and US for the treatment of ADA SCID children. To date, 5 patients aged between 1.2-4.5yrs have been treated, after conditioning with Busulfan i.v. at a single dose of ~5mg/kg. A mean of 5 x 10e6 CD34+ cells/kg were infused with a mean vector copy number (VCN) in the transduced population of 4.2 (range: 2.4-6.1). At a mean follow up of 361 days, there has been significant immunological recovery, with a rise of total T cell and CD4+ counts and normalization in mitogen responses. Integration site analysis shows some expansions but no persistence of expanded clones (reported at ESID meeting 2014 *(Gaspar et al)*).

1.1.9 Insertional Mutagenesis and ADA SCID

To date, no adverse events of clonal proliferation have been reported in over 40 ADA-SCID patients treated with γ-RV in Italy, UK, and USA since 2000.

A major concern has been the vector-related leukaemogenesis observed with a γ-RV of similar architecture in two of the clinical trials for X1-SCID (150, 151). GammaRV preferentially integrate near the regulatory regions of active genes (152); in these early clinical trials the vectors used had intact LTRs (long terminal repeat sequences) with powerful viral enhancer-promoter sequences that are able to drive the expression of not only the therapeutic gene in the provirus, but also the expression of genes surrounding the insertion site. In 5/20 patients in the SCID-X1 trials, the vector integrated near a proto-oncogene (in 4 cases, LMO2), deregulating its expression and contributing to the development of T-cell leukaemia. Moreover, it has been recently documented by *Pignata's* group the involvement of γc in several immune and endocrine pathways, supporting the implication of such a molecule in the control of cell growth and proliferation under physiological or pathogenic conditions (152*bis*). In particular, it has been shown a direct relationship between the amount of γc expression and the proliferative capability of control lymphoblastoid cells (152*ter*), implying a direct involvement of γc in self-sufficient growth in a concentration-dependent manner. The same group demonstrated that the knockdown of γc molecule through short interfering

RNA is able to decrease the cell proliferation rate in malignancies, thus confirming a direct involvement of the molecule as a key player in cell cycle progression (152*quater*).

In a trial for CGD, clonal dominance was observed with γ-RV insertions occurring near the *MDS1-EVI1* locus (153, 154). In a more recent update on GT clinical trial for WAS by *Klein et al*, 7/10 treated patients developed hematologic malignancies (4 cases of T-cell acute lymphoblastic leukemia, 2 primary T-ALL with secondary AML and one acute myeloid leukemia), with hotspots found within *LMO2* and *MDS/EVI1* (16, 155). Integration site analyses have been analysed for the ADA SCID patients that had participated in the TIGET study (145) and in the UK study (unpublished data). Both show a high proportion of insertions occurring in the proximity of gene transcriptional start sites (TSS), with more than one insertion occurring near tumour associated genes, including *LMO2*. However, despite the long-term follow up, no clonal proliferation has been observed in any of the patients. TIGET has carried out a deep analysis of the samples obtained from the patients pre- and post-GT. Firstly, since the insertions in the proximity of LMO2 were of particular concern, the expression of this gene was analysed by Q-RTPCR in purified post-GT granulocyte, CD3+, CD4+ and CD8+ populations from four patients and compared to healthy controls; no significant difference was observed between both groups in any of the populations (145). In a subsequent study, the expression profile of purified CD4+ and CD8+ populations post-GT from three patients was studied by microarray analysis and compared to the profile of age-matched healthy controls; and again no significant differences were observed. T cell clones were isolated from two patients, and the expression of the genes in the 100 kb window around the insertion site was analysed. Approximately 5.8% of the genes analysed were either up or downregulated, but these changes did not translate into proliferative advantage or altered functional responses to mitogens in the individual clones; nor did they reflect onto the gene expression profile of the bulk T cell populations. Nevertheless, none of these clones had insertions near LMO2 (156).

The question remains as to whether the clones with γ-RV insertions near proto-oncogenes are being selected *in vivo*, and if that is the case, why are they not dominant in the ADA-SCID patients. The most recent data from TIGET seems to give an explanation on this point (129). In order to unveil the influence the target cell type and its status has on vector integration and engrafment, the samples pre-infusion and post-GT from the PBL-GT (142) and the HSC-GT (141) studies were analysed for vector

integration distribution. First, comparing the pre-infusion to post-GT datasets in the HSC-GT study, a slight skewing of the T cell and granulocyte populations towards insertions near TSS of genes is observed in the post-GT group, that is not seen in the PBL-GT samples; however the TCR diversity remained polyclonal at the time of collection, indicating no clonal dominance. A number of integration hotspots were identified in the pre-infusion samples for both PBL-GT (transduced CD3+) and HSC-GT (transduced CD34+), but there was little overlap between them. The integrations largely occurred in genes that were highly expressed in the target cell at the time of transduction, many of them cell-type specific. The hotspots were later associated with DNAse hypersensitive sites and histone modifications characteristic of open chromatin, pointing towards a preference for integration within transcriptionally active regions. In addition, the CD3+ retrieved post-GT shared few integration sites with their transduced HSC progenitors, and these integrations were within gene regions that remained transcriptionally active in T cells (129).

In summary, the existence of similar integration sites in the cells from ADA-SCID and SCID-X1 or CGD gene therapy patients is not necessarily indicative of clonal selection due to transformation. The initial integration into CD34+ cells pre GT is influenced by the open chromatin areas in the genome at the time of transduction and these areas are similar for all three diseases. Furthermore, the enrichment for insertions within active gene expression regions in the CD3+ cells post-GT is most probably related to the fact that cells capable of expressing ADA have a selective advantage in a toxic metabolite environment. A number of other factors, such as disease background, the ADA transgene or the kinetics of lineage reconstitution may determine whether sufficient mutagenic events are accumulated for leukaemic transformation (138).

1.2 AIMS

The aim of the present study is to evaluate the long term outcome of 18 patients affected by ADA deficiency treated between 2000 and 2011 with HSC-GT after non-myeloablative conditioning regimen.

Specifically, we compared long-term survival with those of standard bone marrow recipients and focused on immune reconstitution after treatment.

1.3 PATIENTS AND METHODS

1.3.1 Overview of the Clinical Development Program

Eighteen patients have been treated in the context of two pilot studies (AD1117054, N=1 and AD1117056, N=2), one pivotal study (AD1115611, N=12, named *main* study) and a compassionate use program (CUP, N=3). All patients were then enrolled in a long-term F-U study, that allowed long-term assessments beyond the initial follow-up period of each study.

The clinical trials were founded initially by Fondazione Telethon. Following initial encouraging results, in 2010 GSK acquired the licence for the product and began to complete its development towards marketing authorization.

1.3.2 Clinical Studies

The pilot study 1 enrolled 1 subject (Subject 1) in 2000 who was treated with open-label GSK2696273 at Hadassah University in Jerusalem, Israel, with the cooperation of Ospedale San Raffaele s.r.l. Istituto Scientifico San Raffaele (HSR-TIGET) in Milan, Italy. Subject 1 was consented to LTFU from Year 13 onward. Data from this subject are summarized in previous publications (109, 141).

The pilot study 2 contained data from 2 ADA-SCID patients (Subjects 2 and 3) starting in 2001 who were treated with HSC-GT and included follow-up data for 3 years post-treatment at the HSR-TIGET in Milan, Italy. The study protocol is described in a publication (158). Study design and enrollment criteria of the second pilot study were similar to those later defined in the main study (see below).

The main study was an open-label, prospective, non-randomized, historical control, single-center phase 1/2 study conducted at the HSR-TIGET in Milan, Italy. In this study, 12 subjects (Subjects 4 to 15) were enrolled starting in 2002 and treated with HSC-GT, then followed for 3 years post-treatment. Study design is illustrated below.

Three patients (Subjects 16 to 18) received HSC-GT under a named patient program for compassionate use (hereafter referred to as the CUP). The program was initiated in 2010 at HSR-TIGET in Milan, Italy. All subjects had baseline characteristics consistent with the entry criteria for the main study and were followed for 3 years post-treatment.

Subjects 8 and 17 were withdrawn from the program after being treated with GT to receive HLA-matched sibling donor SCT.

After the HSC-GT was in-licensed in 2010, GSK implemented a protocol amendment to the main study that formally extended longer-term follow-up to >3 years, and enrolled subjects after 3 years of post-treatment follow-up in their initial study/program, which included Pilot Study 2 and the CUP. The single subject treated in Pilot Study 1 joined the AD1115611 LTFU study 13 years post-treatment.

1.3.3 Summary of the main phase I-II study

A summary of the characteristics of the main clinical trial as Final Version (date 02/04/2007) are reported below.

Study design

The main study was designed to investigate the safety and clinical efficacy of infusion of autologous CD34 positive cells transduced with a γRV vector in patients affected by ADA SCID in the absence of ERT.

Principal Investigator: Prof. Maria Grazia Roncarolo (Pediatric Clinical Research Unit HSR-TIGET, Milano), then replaced by Prof. Alessandro Aiuti.

Co-Investigators: Dr. Alessandro Aiuti (Pediatric Clinical Research Unit HSR-TIGET, Milano)

Dr. Fabio Ciceri (Hematology and Bone Marrow Transplant Unit - hSR Milano).

Study objectives

1. Evaluation of the safety and the clinical efficacy of gene therapy, in the absence of enzyme replacement therapy.
2. Evaluation of biological activity (engraftment, ADA expression) of ADA transduced CD34+ cells and their hematopoietic progeny.
3. Evaluation of immunological reconstitution and purine metabolism after gene therapy.

Study endpoints

Efficacy endpoints

Primary endpoint

1) Survival

Secondary endpoints (in hierarchical order)

1) Change in the rate of severe infections (defined as infections requiring hospitalization or prolonging hospitalization);
2) One-year change in T-lymphocyte counts (cells/µl);
3) Modification of the "systemic" metabolic defect, analyzed by levels of purine metabolites in RBC;
4) One-year change in the proliferative response to polyclonal stimuli;
5) One-year change in thymic activity (T-cell receptor excision circles);
6) Presence of genetically modified cells in the bone marrow compartment and presence of ≥ 10% genetically modified cells in peripheral blood lymphocytes;
7) Lymphocyte ADA enzyme activity;
8) One-year change in lymphocyte counts (cells/µl);
9) Recovery of physical growth;
10) Need of reintroduction of PEG-ADA (in patients previously treated with PEG-ADA);
11) Antibody response to vaccination.

Safety endpoints

Adverse event (expected or unexpected)

Serious adverse event (expected or unexpected)

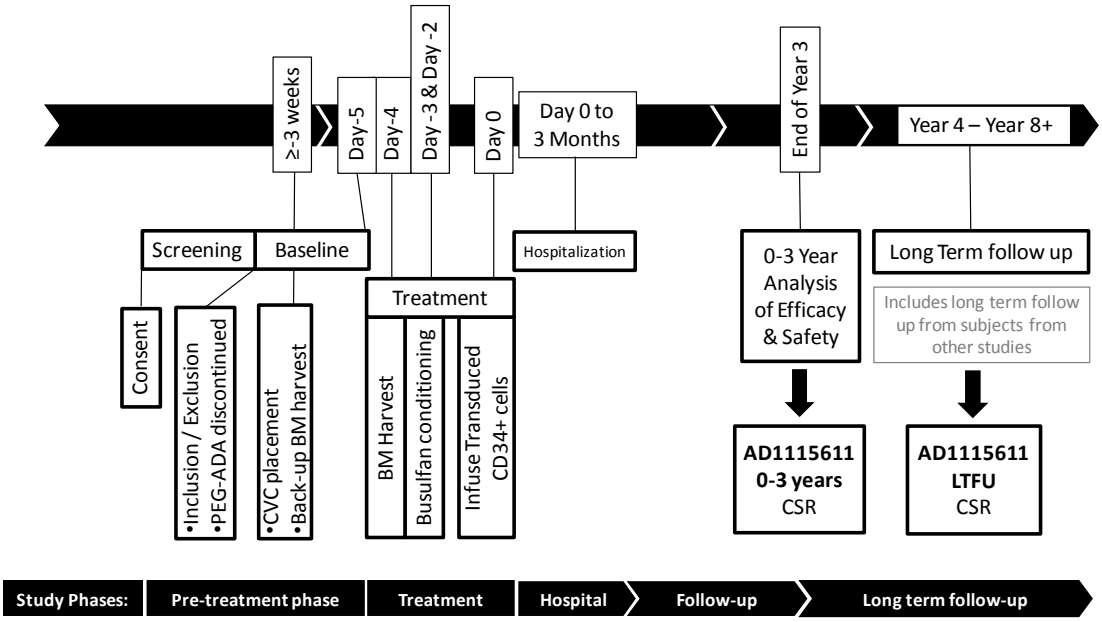
Study phases

Patients were enrolled in the clinical trial after signature of the informed consent by their parents or legal tutors.

After enrollment, they went though the six phases foreseen by the protocol (**Figure 1.3.1**):

1) *Screening phase*, during which the conditions required by the clinical protocol for patients' enrolment were assessed;
2) *Inclusion-Exclusion criteria application*, at the end of the screening phase;
3) *PEG-ADA discontinuation*, applicable for patients who at time of the application of inclusion/exclusion criteria were receiving PEG-ADA;
4) *Baseline phase*, carried from the end of the screening phase to the day before the harvest of stem cells (day -5);
5) *Treatment phase*, from day - 4 to day 0;
6) *Follow-up phase*, during which patients underwent regular follow-up for a period of 3 years after gene therapy, to assess the safety and the efficacy of the treatment. All subjects who completed the 3-year follow-up were offered the opportunity to remain in follow-up for an additional 5 years; thus, the total period of follow-up for each subject from the date of treatment was to be at least 8 years. After in-licensing of the product in 2010, GSK implemented a protocol amendment that extended the study follow-up until marketing approval of the product.

Figure 1.3.1 Study design.



Abbreviations: BM = bone marrow; CSR = clinical study report; CVC = central venous catheter; LTFU = long-term follow-up; PEG-ADA = polyethylene glycol-adenosine deaminase.

Study population

The target study population included patients <18 years old with ADA-SCID, as assessed by ADA enzymatic activity and/or genetic analysis, who lacked an HLA-identical sibling, had received ≥6 months PEG-ADA treatment (except in cases in which PEG-ADA was unavailable or was not considered appropriate treatment), and who had demonstrated failure or intolerance to PEG-ADA therapy. Gene therapy treatment consisted of a single infusion of autologous transduced CD34+ cells after which subjects were followed up for 3 years to evaluate safety and efficacy endpoints. After the 3-year follow-up period, subjects were followed-up annually.

Inclusion Criteria

Patients were enrolled according to the following criteria:

- ADA-SCID patients for whom an HLA–identical healthy sibling donor was not available as suitable bone marrow donor. The decision to treat the patients with alternative transplantation strategies was taken independently, before the

patient was taken into consideration for recruitment into the study;

- Patients of pediatric age (< 18 years of age);
- Patients for whom their legal tutors have signed the Informed Consent;

and at least one of the following criteria:

1) Patients who received enzyme replacement therapy (PEG-ADA) for at least 6 months before enrolment and displayed at least two of the following immune parameter alterations:
   - Absolute lymphopenia (<1500/µl)
   - Absolute T lymphopenia (<1000/ µl)
   - Requirement for IVIg infusion
   - Deficit of serum immunoglobulins (IgM or IgA or subclasses of IgG) or lack of antibody response to vaccination.
2) Patients who received enzyme replacement therapy (PEG-ADA), but were forced to discontinue the drug due to intolerance, allergy, or autoimmune manifestations;
3) Patients for whom enzyme replacement therapy (PEG-ADA) is not a life long therapeutic option (e.g. patients from countries in which the drug is not available).

Exclusion Criteria

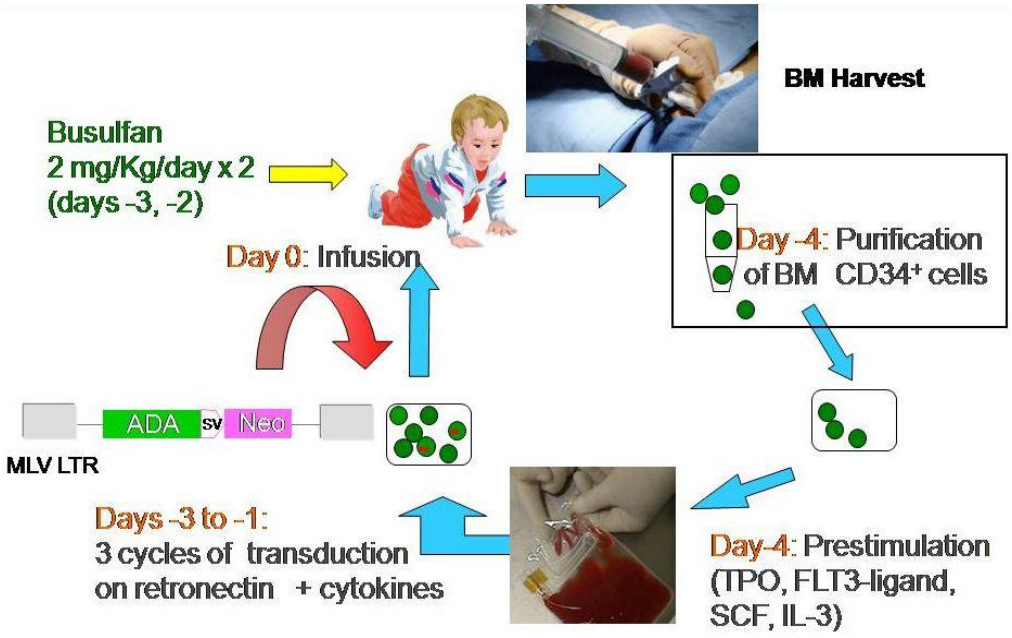
Patients were excluded from the study if met one of the following criteria:

- Patients suffering from HIV infection;
- Patients with history or current malignancy;
- Patients who received a previous gene therapy treatment in the 12 months preceding the enrolment;
- Patients suffering from any other clinical condition that in the Investigator's opinion was dangerous for the patient and would have prevented the good conduction of the experimental treatment, according to requirements.

1.3.4 Treatment design

Treatment design is summarized in **Figure 1.3.2.** Apart from Subject 1, the treatment was performed for all the patients at the San Raffaele Hospital, Milan, Italy.

Figure 1.3.2 Treatment design.



On day -4, patients underwent BM harvest under general anesthesia. Autologous CD34+ cells were then purified, collected and transduced three times with GIADAl retroviral vector and re-infused back to the patients on day 0. On days -3 and -2, patients received conditioning, consisting of iv Busulfan, at a body weight-based and AUC-targeted dose, aimed to achieve significant depletion of endogenous HSPC cells, with limited toxicity.

Note: Busulfan was administered orally in Subject 2 and, partly, in Subject 7 .

1.3.4.1 Medicinal product

The medicinal product is defined as an autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with a retroviral vector that encodes for the human ADA cDNA, to be administered intravenously.

1.3.4.2 Dosage indications

The optimal target dose to be infused iv to patients was 5-10x10^6 transduced CD34+ cells/Kg. Collection of 10-20x10^6 CD34+cells/Kg was recommended to let the patient receive a dose of manipulated cells within the target dose.

1.3.4.3 Preparation to GT: CVC and stem cell back-up

Before treatment, a central venous catheter (CVC) was placed under general anesthesia to allow infusion of required intravenous medications for conditioning, intravenous infusion of transformed cells, and intravenous medications/immunoglobulin and blood products post transplant. The CVCs were maintained for approximately 2 years post-treatment.

At least 3 weeks before gene therapy, the patients underwent bone marrow harvest, to collect stem/progenitor cells as rescue therapy available for future infusion.

Whenever possible, the back-up collection was combined to the implant of CVC or to other diagnostic procedures requiring sedation/anesthesia. The back-up contained at least 1x10^6 CD34+ cells/Kg and was frozen and stored un-manipulated in liquid nitrogen, to be used in case of transplant failure or prolonged bone marrow aplasia.

1.3.4.4 Antibiotic and Antiviral Medication Use

Planned concomitant therapy included pentamidine as first-line *Pneumocystis Jirovecci* prophylaxis from the day before gene therapy until recovery of white blood cell counts, cotrimoxazole as *Pneumocystis Jirovecci* prophylaxis after recovery of blood cell counts, intravenous/oral antibacterial and antifungal prophylaxis according to local standards, acyclovir as Herpes Simplex prophylaxis until complete immunological reconstitution, pre-emptive therapy with ganciclovir or foscarnet according to local standards for subjects who were cytomegalovirus positive, and pre-emptive therapy with rituximab to subjects who were Epstein Barr virus positive and experienced a substantial increase in cellular viral load.

1.3.4.5 Bone marrow harvest

After the patient had taken a bath, bone marrow was collected from the anterior and posterior iliac crests in the operating room under sterile conditions and using general anesthesia or deep sedation, on day –4. The bone marrow collection procedure was described in an internal SOP and was performed by trained physicians.

Collection was performed through multiple aspirations with syringes connected to bone marrow aspiration needles. The bone marrow material was transferred from the syringe to a sterile bag containing anticoagulant. During the procedure, samples were taken to assess the number of nucleated cells using a hemogram. The number of CD34+ cells were also assessed by flow cytometry.

The total volume of the required amount of bone marrow was estimated according to the content of bone marrow CD34+ cells determined at the start of the collection. On average, approximately 20-30 ml/kg patient body weight was collected to ensure sufficient cells were present to support production of gene corrected CD34+ cells. When the bone marrow collection was completed, it was packaged in a closed sterile transfer pack appropriate for blood or marrow products, sealed and labelled and then transferred in a biohazard container from the research nurse or a physician to the manufacturing facility (MolMed S.p.A.).

Hemoglobin values < 7.0 g/dl were corrected by irradiated and filtered red blood cells transfusion.

1.3.4.6 Conditioning regimen

To prepare the bone marrow compartment for HSC engraftment, the chemotherapy agent busulfan was given as non-myeloablative pre-conditioning prior to GT. This regimen was selected based on SCT engraftment outcomes showing enhanced gene marking with a non-myeloablative approach (109). The busulfan dosing was 2 mg/kg/day on days -3 and -2 (total final dose 4mg/Kg). If the administration of i.v busulfan was not possible (e.g. occlusion of the CVC), busulfan was administered orally (4 doses of 0.6 mg/Kg).

Actually, the used dose represents approximately 25% of the typical dosage used in a myeloablative regimen, which would usually include additional chemotherapy agents at high doses. The short half-life of busulfan (2.5 to 3 hr) was sufficient for drug

clearance prior to administration of the gene therapy dose. After the first administration of busulfan, the plasmatic levels of the drug (Area under the curve, AUC) were measured and the dose was adjusted if needed for the second day of administration, based on actual exposure from the first day.

1.3.4.7 Infusion of autologous gene modified CD34+ cells

On day 0, patients had been prepared for the infusion of the transduced cells. Before it, all other iv drugs had been interrupted and a premedication with an antihistaminic drug (chlorphenamine) had been administered. Approximately 15-30 minutes afterwards, transduced cells were infused iv through the CVC in about 20 minutes. At the end of infusion, normal saline solution was used to wash the syringe previously containing the medicinal product and the CVC line. Vital signs (BP, HR, SO2) and symptoms had been monitored before infusion, then every ten minutes during it and every hour for 3 hours thereafter.

1.3.5 Assessment of safety

All AEs /ADRs of any severity and not considering the correlation with the medicinal product, were recorded on the corresponding page(s) included in the Case Report Form (CRF) and, when possible, symptoms were assembled as a single syndrome or diagnosis. Date of onset, maximal intensity, action taken with respect to medicinal product, corrective therapy given, outcome and investigators' opinion on a possible correlation between by the study medicinal product and AEs/ADRs were clarified. In the case, the patient had to be followed up until clinical recovery was complete and laboratory results had returned to normal, or until progression had stabilized.

Toxicity grades of Adverse Events were defined based on the NIH/NCI Common Terminology Criteria for Adverse Events (CTCAE, version 4.0), as follows:

- Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.

- Grade 2: Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living.
- Grade 3: Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activities of daily living.
- Grade 4: Life-threatening consequences; urgent intervention indicated.
- Grade 5: Death related to AE.

Safety assessments were the following:

- AEs and SAEs
- Hematologic parameters;
- Clinical chemistry assessments, which include liver function tests
- Vital signs
- Electrocardiogram (ECG);
- Physical examination;
- Specialist examinations (ophthalmologist, neurologist, psychologist);
- Instrumental tests (abdominal ultrasound scan, auditory evoked potential, tympanometry/audiometry and, for children over 5 years of age, respiratory functional tests);
- Urinalysis;
- Bone marrow morphology and bone marrow immunophenotype
- Replication competent retrovirus testing.

The AEs were transferred to GSK and assigned to one of the following 6 phases of the study:

- Pre-treatment: between screening visit but and Day -4 (excludes Subject 1);

- Treatment phase: between Day -4 up to and including the date of gene therapy (excludes Subject 1);
- 3-month hospitalization: between the day after the date of gene therapy and up to and including the end of the 3-month visit F-U (excludes Subject 1);
- 3 months to 3 years follow-up: starting between the 3-month visit F-U up to and including the end of the 3-year visit F-U (excludes Subject 1);
- 4 to 7 years follow-up: starting after the end of the 3-year visit up to and including the end of the 7-year visit F-U.

The numbers of pre-treatment SAEs are likely under-estimated, as AEs were not formally collected prior to the start of treatment and information collected on the medical history and concomitant diseases CRF pages has been used to determine pre-treatment SAEs that led to hospitalization, were life-threatening or led to death.

1.3.6 Assessment of Efficacy

1.3.6.1 Efficacy Endpoints

The following efficacy endpoints were evaluated:

1) Survival
2) Intervention-free survival
3) Severe infection rate
4) Lymphocyte counts
5) Thymic activity (TREC)
6) V-beta repertoire in T cells
7) Lymphocyte proliferation
8) Serum immunoglobulins IVIG use post-gene therapy
9) Vaccination responses
10) Physical growth
11) Presence of gene modified cells
12) Requirement for post-treatment PEG-ADA therapy

1.3.6.2 Efficacy Evaluations and statistical analysis

The reported data of the clinical development of the gene therapy medicine have been retrospectively collected from Glaxo-Smith-Kline (GSK) and the analyses of the data performed by GSK.

Assessment of survival was presented as Kaplan-Meier curve, with a 95% CI. The survival rate following GT was compared with the published 67% overall survival rate in patients treated with conventional HLA-matched hematopoietic SCT from a MUD (58).

All available data except for Subject 1 data were included in the analysis for the intervention-free survival. Time to event (intervention or death) was presented using a Kaplan-Meier Survival curve.

Severe infections and durations were identified from prior medical history and post-therapy adverse events (AEs). Infection rates were estimated pre- and post-treatment (up to 8 years) as the number of severe infections (defined as infections leading to or prolonging hospitalization) per person-year of observation. The 3-month hospitalization period post-treatment was not considered in the analysis.

Changes from baseline for cell populations numbers and function over time were described by summary statistics and appropriate plots. Mean changes from baseline at each year post-treatment (up to 8 years) were assessed using a MMRM analysis. A summary of proportion of responders determined by CD3+, CD3+CD4+ and CD19+ cell counts used a denominator based on non-missing responses at each time point.

Physical growth of individual subjects was assessed by height and weight over time, presented by gender. Height and weight for all subjects was compared with WHO growth charts. Numbers of subjects shifting to above or below the 5^{th} percentile for height and weight at baseline and at Years 1, 3, 5 and 8 were presented in appropriate charts for each gender.

Percentage of subjects with genetically-modified cells was summarized with 95% CIs, and longitudinal profiles over time were described by summary statistics and plots (both original and log-transformed data). Percentage of ADA responders was presented with 95% CIs, and longitudinal profiles and changes from baseline for lymphocyte ADA enzyme activity over time were described by summary statistics and plots.

Percentages of subjects reaching adequate systemic metabolic detoxification each year post-treatment were summarized separately for peripheral blood and bone marrow. The denominator for percentages was based on non-missing responses at each time point. The percentage at each time point was compared with a 10% reference by means of a 1-sample proportion test (1-sided test) with a 95% CI for the proportion.

Number/percentage of subjects who received post-treatment PEG-ADA, and time to receipt of ≥3 months of continuous PEG-ADA, were summarized.

To evaluate CNS abnormalities, a customized query for neurologic, cognitive, and hearing impairment events was conducted that included a summary of subjects who experienced events in these following categories of system organ classes: Nervous System, Psychiatric Disorders, Ear and Labyrinth Disorders, and General Disorders and Administration Site Conditions.

Exploratory analyses of baseline predictors of efficacy were conducted utilizing data during the 0-3 years of follow-up from the main study, Pilot Study 2, and CUP. The following covariates were considered as potential predictors of efficacy: age at gene therapy, CD34+ cells/kg and CD34+ cells/kg*VCN, and baseline values for lymphocyte (CD3+) counts in peripheral blood, TREC counts, dAXP concentration in peripheral blood, and body mass index (BMI). The impact of these potential baseline predictors was assessed on intervention-free survival during the first 3 years, CD3+ cell counts in peripheral blood, and RBC dAXP levels in peripheral blood.

For the intervention-free survival over the first 3 years, box plots were produced for each potential predictor comparing baseline covariate (y-axis) against categories of intervention-free survival (x-axis). Given the limited number of subjects, no formal statistical analysis was performed. For CD3+ count and dAXP levels in RBC at the 3-year visit, scatter plots were produced with endpoint of interest (y-axis) versus baseline characteristic of interest (x-axis). Pearson correlation coefficients were included.

Subject 1, the single subject enrolled and treated in Pilot Study 1, contributes only the date of gene therapy from their original study for the purposes of the integrated efficacy analyses (to enable calculation of their duration of follow-up and survival), as there is no formal protocol available for Pilot Study 1.

1.3.7 Laboratory studies

These investigations were performed in HSR-TIGET laboratories.

Frequency of transduced cells

Cell subpopulations were purified using antibody-coated microbeads or FACS sorting. The frequency of transduced cells and vector copy number were determined on genomic DNA by quantitative PCR analysis for NeoR vector sequences, normalized for DNA content *(Aiuti A et al, NEJM).*

Immunological Studies

All immunostaining and flow cytometry were performed as described (141).

In vitro T-cell responses to mitogens and antigens were measured by thymidine incorporation in proliferative assays (141).

Specific antibodies were analyzed by ELISA, following patients' immunization with toxoid vaccines, conjugated or bacterial polysaccharide antigens, or measles vaccine (159).

TREC were measured in PB T cells by PCR2 and the TCR Vβ repertoire was analysed by FACS analysis using a mix of directly conjugated antibodies corresponding to 24 different TCR Vβ specificities (IOTest Beta Mark kit; Immunotech, France) and by spectratyping analyses (160).

Biochemical studies. ADA activity in cell lysates was measured by an adenosine to inosine conversion assay, followed by high-performance-capillary-electrophoresis (HPCE) (40). Total adenine ribo-(AXP) and deoxyribonucleotides (dAXP) in erythrocytes were measured by HPCE (40).

The systemic metabolic defect was analyzed by measuring levels of purine metabolites in bone marrow and peripheral blood, where levels of dAXP in RBCs have been correlated with severity of disease (56).

1.4 RESULTS

1.4.1 Treated patients characteristics

As of May 2014, a total of 18 subjects were treated with HSC-GT gene therapy.

The features of all 18 subjects treated with GT (sex, race, country of origin, age at treatment, F-U duration) at the time of GT are summarized in **Table 1.4.1**.

All the subjects were affected by SCID due to ADA deficiency with early-onset manifestations.

The median duration of follow-up is 6.94 years (range: 2.6 to 13.4). No deaths have been reported. Sixteen subjects are currently in long term clinical follow up with long term follow up data available for 14 of these.

1.4.1.1 Subject Demographics

The approximate age at the time of ADA-SCID diagnosis ranged from <1 month to 1.25 years. Most subjects were white and 61% were male (11 male and 7 female). All subjects lacked a sibling HLA matched stem cell donor and were either failing to respond adequately to PEG-ADA or did not have access to it. Patients' country of origin included the EU, Middle East, Africa, North America and South America (**Table 1.4.1**).

Consanguinity of parents was reported for 8 patients (Subjects 4, 5, 6, 8, 10, 13, 17 and 18).

The median subject age at the time of gene therapy was 1.7 years (range: 0.5 months to 6.1 years) **(Table 1.4.2)**. All subjects received busulfan reduced intensity conditioning prior to gene therapy**.**

Table 1.4.1 Summary of Subjects Treated in GSK2696273 Clinical Program

Subject	Sex	Race	Clinical Study	Prior SCT or PEG-ADA, duration	Age at gene therapy, yrs	GT treatment date	Busulfan dose, mg	HSC-GT CD34+ cells x10^6/kg	VCN of product	Follow-up duration, yrs
1[#]	F	White/Arabic	Pilot 1 (Israel)	None	0.6	07 Sept 2000	Not reported in CRF *	8.5	2.28	13.4
2	F	White	Pilot 2 (Tiget)	Haplo-SCT	2.4 5.0	09 Mar 2001 17 Oct 2003[§]	24.0 (oral) None[§]	0.9 2.1	NA 2.15	13.1
3	M	White	Pilot 2 (Tiget)	Haplo-SCT	1.0	04 May 2002	17.4	6.7	0.85	11.9
4	F	White/Arabic	Main	Haplo-SCT & PEG-ADA (2 mo)	1.9	19 Oct 2002	17.0	3.8	NA	11.6
5	F	White	Main	PEG-ADA (15 mo)	1.6	26 Mar 2004	20.0	9.6	1.89	9.9
6	M	White	Main	PEG-ADA (65 mo)	5.6	12 Nov 2004	14.5	9.46	1.05	9.0
7	M	White	Main	PEG-ADA (13 mo)	1.5	11 Nov 2005	17.4	9.0	0.82	7.0
8[$]	F	White	Main	PEG-ADA (32 mo)	2.8	17 Feb 2006	15.4	10.6	0.12	2.3
9	M	White	Main	PEG-ADA (10 mo)	1.4	20 Sep 2006	20.0	13.6	0.57	7.0
10	F	White/Arabic	Main	Haplo-SCT & PEG-ADA (11 mo)	1.8	17 Nov 2006	20.0, 15.0[$]	10.7	0.35	6.9
11	M	White	Main	PEG-ADA (8 mo)	1.6	09 Mar 2007	16.0	6.35	0.17	7.0
12	M	White	Main	PEG-ADA (12 mo)	1.3	19 May 2007	24.0	11.5	0.14	6.2
13	M	Asian	Main	PEG-ADA (1 mo)	0.5	28 Jun 2007	10.0, 3.0	18.15	0.06	6.2
14	M	White	Main	PEG-ADA (71 mo)	6.1	30 May 2008	44.0	5.97	0.54	5.1
15	F	White/Arabic	Main	PEG-ADA (12 mo)	2.5	27 Jun 2008	22.80	5.94	0.38	5.0
16	M	AA	CUP	PEG-ADA (23 mo)	2.3	26 Mar 2010	34.0	6.91	0.17	3.2
17[&]	M	AA	CUP	PEG-ADA (7 mo)	0.7	16 Apr 2010	16.0	12.95	0.24	3.7
18	M	White/Arabic	CUP	PEG-ADA (24 mo)	2.1	20 May 2011	24.0	9.9	0.11	2.6

Abbreviations: AA = African-American/African heritage; F = female; M = male; PEG-ADA = polyethylene glycol adenine deaminase; haplo-SCT = haplo-identical stem cell transplant; NA = not available; VCN = vector copy number; HSC-GT= hematopoietic stem cells-gene therapy.

[#] Subject 1 data from Years 0 to 13 limited to the date of gene therapy (for duration of follow-up and survival analysis) and data collected in LTFU (Year 13 onwards).

* i.v. 2 mg/kg/day for 2 days *(Aiuti et al, 2009)*.

[§] Subject 2 received a second dose of gene therapy product that did not include busulfan pre-conditioning.

[$] Subject 8 withdrew from the main study on 08 June 2008 (reason: investigator discretion) when she became a candidate to receive an allogeneic sibling donor SCT.

[&] Subject 17 withdrew from LTFU prior to the Year 4 visit (reason: investigator discretion) when he became a candidate to receive an allogeneic sibling donor SCT; no LTFU data were reported.

Table 1.4.2 Summary of Demographic Characteristics at Baseline/Date of GT

Demographic	**N=18**
Age at gene therapy (years), n	18
Median (min, max)	1.70 (0.5, 6.1)
Sex, n	18
Female, n (%)	7 (39)
Male, n (%)	11 (61)
Height (cm), n	14
Median (min, max)	81.5 (70, 114)
Weight (kg), n	17
Median (min, max)	10.2 (6, 22)
Race, n (%)	18
White – White/Caucasian/European Heritage	10 (56)
White – Arabic/North African Heritage	5 (28)
African American/African Heritage	2 (11)
Asian – Central/South Asian Heritage	1 (6)

1.4.1.2 Subject Baseline Disease Characteristics

The majority of subjects (82%) experienced severe infections pre-GT, defined as infections that lead to hospitalization or prolongation of hospitalization, and most of these subjects had multiple occurrences (collected retrospectively). Overall, 40 events were registered, with an infection rate of 1.17 per person-year of observation.

Apart from infections, 17 subjects reported medical conditions ongoing at screening (**Table 1.4.3**).

Table 1.4.3 Summary of Medical Conditions Ongoing at Screening in Two or More Subjects

Medical condition	N=17
Any condition, n (%)	17 (94)
Combined immunodeficiency	17 (94)
Failure to thrive	9 (53)[a]
Nuclear MRI abnormal	5 (29)
Verbal delay/Speech disorder	4 (23)
Sensorineural hearing loss	2 (11)
Phimosis	2 (11)
Food aversion	2 (11)
Psychomotor retardation	2 (11)

MRI = magnetic resonance imaging

a. Subject 1 also had a clinical history of failure to thrive (Aiuti, 2009).

After combined immunodeficiency, failure to thrive, one of the main characteristic of patients with ADA-SCID (161), was the most frequently reported ongoing medical condition at Screening, reported in Subjects 1, 2, 3, 4, 5, 6, 7, 8, 10 (109), and 11.

CNS abnormalities are also common in ADA-SCID patients. Four subjects in total presented at screening with developmental delay (Subject 6, 7, 8, 11) with impairment in one or more social or psycho-motor abilities (i.e. attention deficit/hyperactivity disorder, communication disorder, speech disorder) (109). Two subjects had sensorineural hearing loss (Subject 8, 14) and two VEP (Visually Evoked Potentials) prolonged latency (Subjects 11 and 15).

Additional neurologic or central nervous system (CNS) abnormalities reported in individual subjects included one subject with mental retardation (Subject 14), one with left hypoacusia (Subject 6), one with generalized hypotonia (Subject 11), and one with hyperactivity (Subject 16).

MRI alterations were reported in 5 patients (Subjects 7, 8, 11, 16) and mild EEG (electroencephalography) was reported in one (Subjects 16) (109).

Other findings ongoing at Screening were hepatic abnormalities in 1 subject (Subject 2) with increased alkaline phosphatase, aspartate aminotransferase [AST], and lactate dehydrogenase [LDH] and 1 subject (Subject 8) with hepatomegaly. Subject 8 also had chronic autoimmune haemolytic anemia.

1.4.1.3 Prior Bone Marrow Transplantation

Four subjects had previously received an unsuccessful stem cell transplant from a haploidentical donor:

- Subject 2 approximately 1 year 10 months prior to her first GT;
- Subject 3 approximately 7 months prior to GT;
- Subject 4 approximately 13 months prior to GT;
- Subject 10 approximately 17 months prior to GT.

1.4.1.4 Prior Enzyme Replacement Therapy

Fifteen subjects had received ERT with PEG-ADA. Among these, 10 subjects were receiving PEG-ADA at the time of the screening visit; PEG-ADA was discontinued 10 to 22 days before gene therapy. Subjects 1, 2, and 3 had not received prior PEG-ADA as it was not available in their country.

1.4.2 Exposure to Busulfan and treatment with Gene Therapy

All subjects received busulfan pre-conditioning before GSK2696273 treatment administration (**Table 1.4.1**). Busulfan was administered at 2 mg/kg/day intravenously (except as noted below), divided into 4 doses of 0.5 mg/kg for a total final dose 4 mg/kg on study days -2 and -3. Doses were reduced if plasma levels exceeded 4000 ng/ml*h, as measured after the 1st and 5th doses. Most subjects received 2 doses and the mean daily dose was 20.6 mg (range, 6.5 mg to 44.0 mg).

One subject (Subject 2) received HSC-GT treatment on 2 occasions, 2 years and 7 months apart (**Table 1.4.1**). Before the first infusion of gene-corrected cells, this subject received oral busulfan pre-conditioning. The subject received a low dose of transduced bone marrow CD34+cells (0.9 x 10^6/kg) due to the low percentage of CD34+ cells at the time of bone marrow harvest. As the subject remained lymphopenic, PEG-ADA was

administered for 7 weeks, starting 2 years and 5 months after first gene therapy, in order to increase cellularity for a second bone marrow harvest. A second gene therapy procedure (2.1 x 10^6/kg of transduced CD34+ cells) without busulfan pre-conditioning was then performed, 3.5 weeks after PEG-ADA had been discontinued.

Subject 7 received the last 2 doses of busulfan as 2 mg/kg/day via oral administration. Subject 6 did not receive busulfan on the second day of the regimen due to high exposure from the first administration.

Subject 10 received busulfan without gene therapy due to a mycoplasma contamination of the bone marrow during the transduction process and the gene therapy product could not be released. Consequently, the patient was not treated, the bone marrow back-up was re-infused and a single dose of granulocyte colony stimulating factor (G-CSF) was also administered; PEG-ADA was re-started. Then, a second busulfan regimen was given when a second dose of GT was transduced and successfully infused.

Among all treated subjects, gene therapy product was administered at a median dose of 9.46x10^6 CD34+ cells/kg (range: 0.9 to 18.15x10^6 CD34+ transformed cells/kg) in a median volume of 26 mL (range: 10 to 40 mL); the median number of total nucleated cells was 23.10 x10^6 (range: 2.4-37 x10^6) (**Table 1.4.4**). The lowest exposure to date was in Subject 2, who received a further treatment later on

Table 1.4.4 Summary of Gene Therapy Treatment

Parameter	Gene therapy (N=17)
Total volume infused (mL)	
Mean (SD)	26.18 (7.32)
Median (range)	26.00 (10.0-40.0)
Total nucleated cells (x 10^6)	
Mean (SD)	21.72 (7.73)
Median (range)	23.10 (2.4-37.0)
Number of transduced[a] CD34+ cells/kg (x 10^6/kg)	
Mean (SD)	8.94 (4.04)
Median (range)	9.46 (0.90-18.15)

Vector specific PCR on colony forming cells (CFC) revealed an average vector copy number (VCN) per genome in CD34+ cells ranging between 2.28 (Subject 1) and 0.06 (Subject 13) (**Table 1.4.1**).

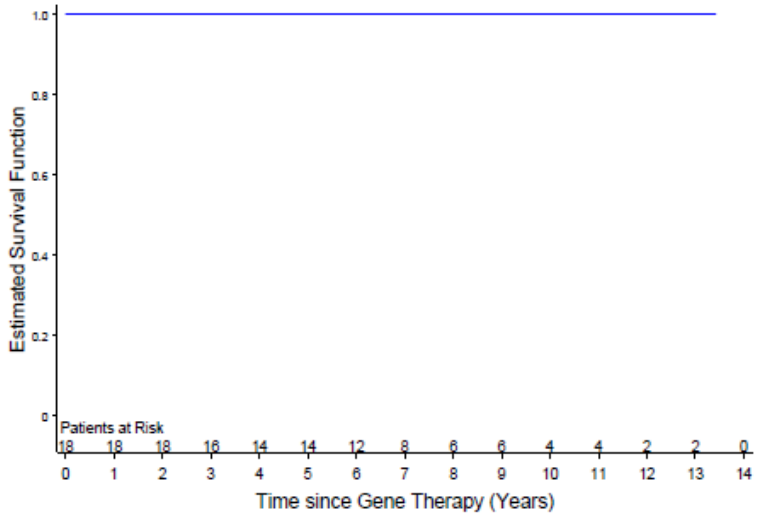
1.4.3 Efficacy outcomes

1.4.3.1 Survival outcomes

1) Survival

A 100% survival rate has been observed for all subjects (N=18) who received HSC-GT treatment, with a median follow-up time of approximately 7 years (**Figure 1.4.1**). This survival rate exceeds the 67% overall survival rate of patients who received a MUD hematopoietic SCT after similar median duration of follow-up (58).

Figure 1.4.1 Survival Kaplan-Meier Plot Showing 100% Survival



Note: Blue line depicted at estimated survival of 1.0 represents 100% survival in the population.

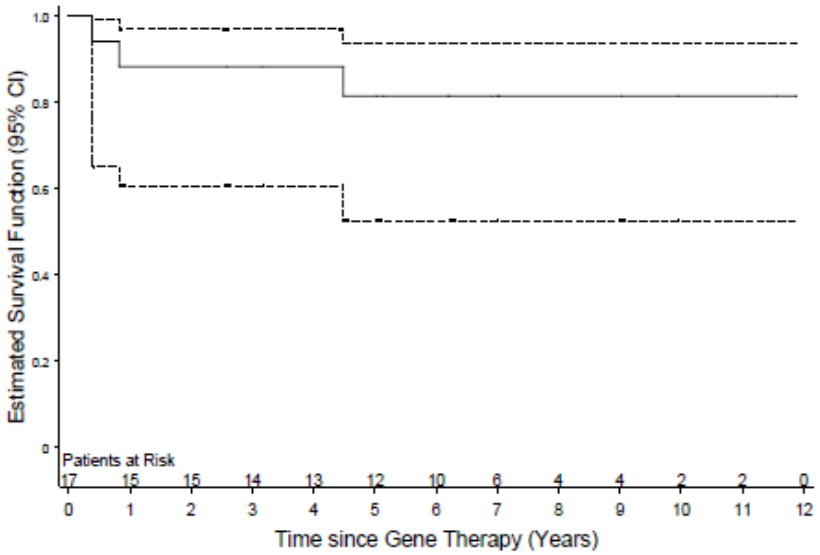
2) *Intervention-Free Survival*

Intervention-free survival was defined as survival without post-gene therapy events of PEG-ADA use for a continuous period of ≥3 months, SCT, or death (**Figure 1.4.2)**. No deaths have occurred. Overall, 82% intervention-free survival rate is observed.

Subject 2, 8 and 17 required continuous PEG-ADA post-gene therapy. Among them, Subject 2 had received a low cell dose and had a poor immune reconstitution; PEG-ADA was administered for 7 weeks in preparation to the second gene therapy, performed without busulfan pre-conditioning and leading to the lack of the engraftment of gene modified cells.

Subject 8 and Subject 17 received GT doses within the therapeutic range, but were considered as treatment failures due to the need for long term (> 3 months) post-treatment PEG-ADA; both of them received post-gene therapy HLA-matched sibling donor SCT when a sibling was born.

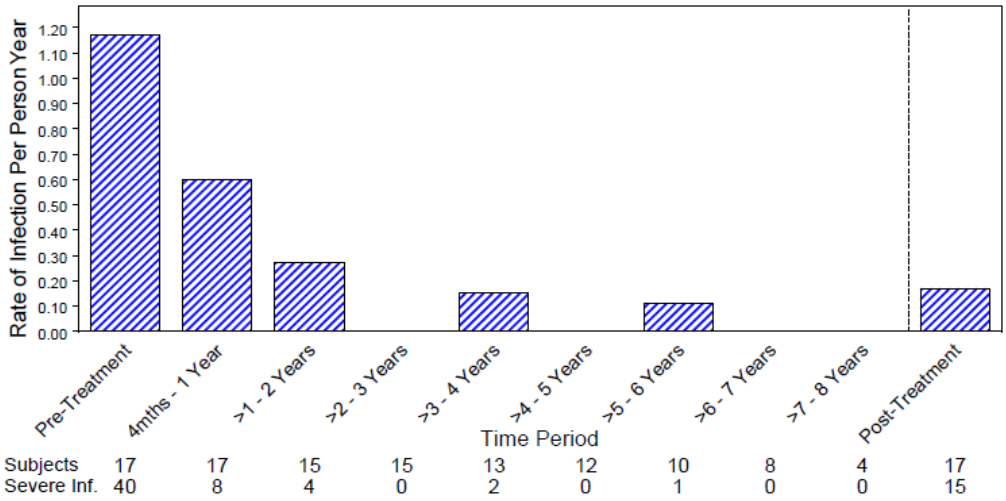
Figure 1.4.2 Survival Kaplan-Meier or Intervention Survival



1.4.4 Severe Infections

Change in the rate of severe infections was observed post-gene therapy (0.26 per person-year - 3 year follow-up) when compared with the pre-gene therapy period (1.17 per person-year) (**Figure 1.4.3**).

Figure 1.4.3 Rate of Severe Infections per Person-Year of Exposure by Year.



Despite the under-reported pre-gene therapy infections, a decrease of post-gene therapy severe infection rates each year following gene therapy is evident.

A total of 15 severe infections were reported after HSC-GT treatment (compared to 40 event pre-treatment) mostly (12/15 events) occurring during the 3-year follow-up, as a common complication of partial immune reconstitution (**Table 1.4.5**). Following gene-therapy, the majority of subjects had only one occurrence of severe infection.

The most frequently post-treatment severe infections were device-related infections (n=5) and gastroenteritis (n=3). Three of the device-related infections were reported in a single subject (Subject 10). Of note, 2 subjects reported varicella infection (Subjects 4 and 5) and 1 subject had *Staphylococcal* sepsis (Subject 16).

The remaining infectious SAEs were respiratory tract infections (n=1), meningitis (n=1), pneumonia (n=1) and pyoderma (n=1).

The rate of severe infections declined in LTFU from Years 4 to 8. Four severe infections were reported in 3 subjects in the LTFU. There were no severe infections during Year 3. Two severe infections were reported between 4 and 5 years after gene therapy (pneumonia in Subject 11 and pyoderma in Subject 13), decreasing to 1 severe infection between 6 and 7 years (varicella in Subject 4), and 1 between 10 and 11 years after gene therapy (pneumonia in Subject 3). In addition, Subject 1 had a severe urinary tract infection at Year 13. All severe infections resolved (**Table 1.4.5**).

Two of the infectious SAEs were Grade 4, as Staphylococcal sepsis and Pneumonia.

Table 1.4.5 Summary of Severe Infections Pre- and Post-Gene Therapy

	N=17	
	Pre-GT	**Post-GT**
n	17	17
Number of patients with events, n (%)	14 (82)	10 (59)
Number of events		
Total	40	15
4 months to 3 years follow-up		12
4 to 8 years follow-up		3
Person-years of observation (free from infection)		
Total	34.30	89.23
4 months to 3 years follow-up		45.81
4 to 8 years follow-up		43.42
Rate of infection[a]		
Total	1.17	0.17
4 months to 3 years follow-up		0.26
4 to 8 years follow-up		0.07
Number of occurrences per patient, n (%)		
n	14	10
1	4 (29)	7 (70)
2	4 (29)	1 (10)
≥3	6 (43)	2 (20)

Abbreviations: GT = gene therapy.

Note: Only data collected prior to PEG-ADA intervention (≥3 months of treatment with PEG-ADA) are included.

a. Rate of infection estimated as number of infections over person-years of observation (free from infection)

1.4.5 Immune Reconstitution

Evidence of immune reconstitution after gene therapy was measured by changes over time in peripheral lymphocyte counts (T cells, B cells, and NK cells), thymic activity (TRECs), peripheral T cell function (proliferation in response to polyclonal stimuli, diversity in V-beta repertoire), and B cell function (assessed indirectly by vaccination responses and IVIG use during follow-up).

1.4.5.1 Immune Cell Counts

Lymphocytes and CD3+ T cell counts significantly increased compared to baseline from Year 1 post-treatment. The increase was maintained throughout the duration of follow-up (**Figure 1.4.4**). There were also sustained increases in CD4+ T cell (**Figure 1.4.5**) and CD8+ T cell subsets (**Figure 1.4.6)** after gene therapy, in particular from Year 1 onwards. CD4+ CD45RA+ naïve T cells followed the same pattern.

Differently, changes from baseline were variable for CD19+ B cells and CD16+ CD56+ NK cells, with counts for both cell types decreasing from baseline to Year 1 and then increasing above the Year 1 counts from Year 2 onwards, achieving levels above baseline by Year 6 (for CD16+ CD56+ NK cells) or Year 8 (for CD19+ B cells) (data not shown).

Figure 1.4.4 Increased CD3+ cells (10^6/L) after Gene Therapy

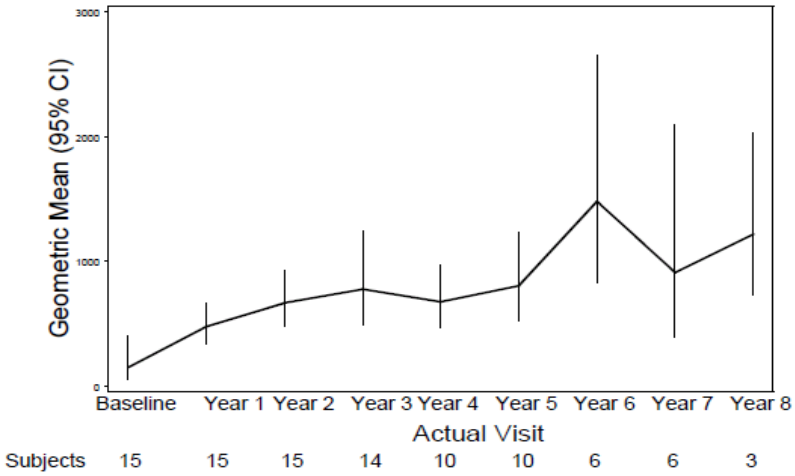


Figure 1.4.5 Increased CD3+CD4+ cells (10^6/L) after Gene Therapy

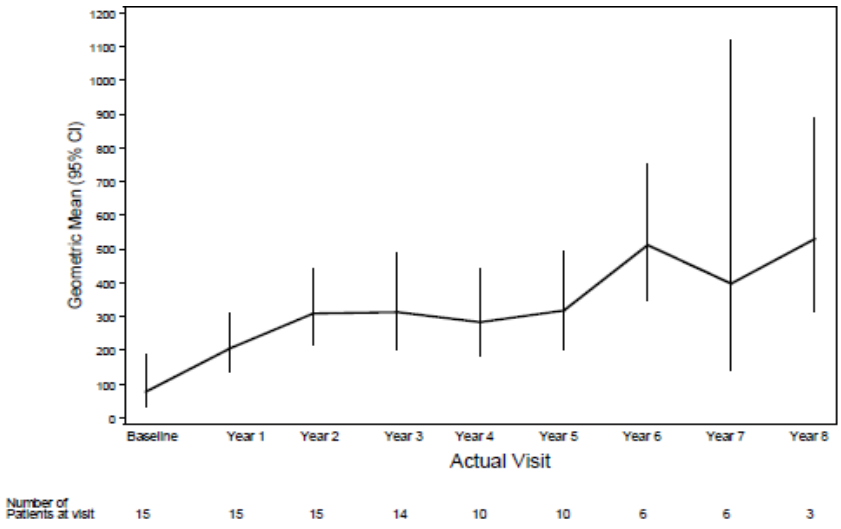
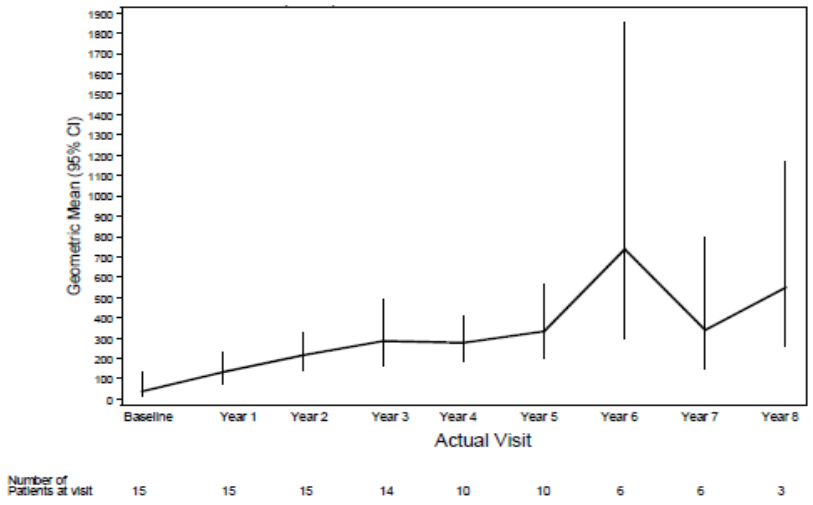


Figure 1.4.6 Increased CD3+CD8+ cells (10^6/L) after Gene Therapy



An exploratory analysis was conducted to determine the proportion of responders at each time point. The subjects were qualified as T cell responders (CD3+ CD4+ count >300x10^6 cells/L and/or CD3+ >1000x10^6 cells/L) or B cell responders (CD19+ count >100x10^6 cells/L) based on cell counts in whole venous peripheral blood. Selection of the cut-off values to define responders was based on criteria reported by Hassan *et al* in patients receiving conventional SCT (58).

At the 6-month visit there were 0/16 subjects who were responders, but by Year 1 (n=15) between 25 to 50% subjects were B cell and T cell responders. By Year 3 (n=14), approximately half of subjects were B cell and T cell responders. By Year 5 (n=10), B cell response was 40%, a level that was generally maintained through Year 8. The proportion of CD3+ CD4+ T cell responders in Year 5 was 80%, and this response level was maintained through Year 8. This trend was generally reflected in the CD3+ T cell responder analyses from Year 5 onwards.

1.4.5.2 Immune Cell Function

1) T Cell Function

- *Thymic Activity*

TRECs in peripheral blood lymphocytes were increased with respect to baseline from Years 1 to 3 post-treatment, and gradually declined at Years 5 and 8, according to the decrease in thymus size and activity that can be expected in older children (162) (data not shown).

The increase in TRECs observed beginning at Year 1 after gene therapy is in line with increased T cell counts from Year 1 onwards and, remarkably, with increased peripheral CD4+ CD45RA+ naïve T cells, as a product of thymic selection and successful recombination of V-beta T cell receptor chains in thymocyte precursors. It also is in line with the decline in severe infection rates from Year 1 onwards after gene therapy, suggesting an improvement of the T cell mediated immune system following gene therapy.

- *T Cell Receptor Repertoire*

The diversity of T cell receptor V-beta chains was evaluated as a measure of T cell polyclonality.

In the main study population, the majority of subjects (7/12) had evidence of polyclonal V-beta chains, although only 4 subjects had reports of a normal V-beta repertoire (polyclonal V-beta chains within the normal range) (data not shown).

The majority of subjects for whom LTFU was available, including subjects from the pivotal study and both pilot studies, had evidence of a normal V-beta repertoire at one or more LTFU visits. Most subjects had presented prior to gene therapy with a polyclonal T cell receptor V-beta repertoire that included some V-beta chains above and some below the normal range.

This was expected in a population of patients with ADA-SCID who were being treated with PEG-ADA; the detection of low numbers of some V-beta chains while

receiving PEG-ADA reflects that the immune system is often not fully reconstituted with PEG-ADA treatment (106).

A diverse repertoire of T cells emerging from the thymus corresponds with other evidence of normal function, including proliferative response, post-treatment decline in severe infection rates, and positive response to routine childhood vaccinations.

- *Peripheral T Cell Proliferation*

From Year 1 onwards the mean and median results in the overall population showed robust proliferation in response to stimulation to both anti-CD3 and PHA polyclonal stimuli, increased over baseline (data not shown).

The increases in T cell counts from baseline in conjunction with these data on proliferative capacity suggest that the increased cell numbers observed in the periphery represent functional T cells capable of clonal expansion.

2) B Cell Function

Despite B cell counts remained low and in some cases decreasing, functional immune protection was demonstrated by assessment of immunoglobulin levels over baseline, the discontinuation of IVIG supportive treatment after receiving gene therapy, and vaccine responses.

- *Immunoglobulins*

Most subjects in the main study showed acceptable levels of gamma globulin via serum protein electrophoresis, and all subjects enrolled in the LTFU had polyclonal serum antibody chains from Year 4 onwards. No serum monoclonal protein was detected, suggesting that the gamma globulins present were polyclonal.

There was a decline in the IVIG substitutive use as LTFU progressed in Years 4 to 8 after gene therapy and this provides evidence for functional B cell and immunoglobulin production in the periphery.

- *Requirement for IVIG Therapy*

All LTFU 15 patient were receiving IVIG treatment before GT and were maintained on IVIG after GT in the first months after treatment. Six patients stopped IVIG during 0-3 years period. Five patients received IVIG during LTFU but stopped before the cut off data. Four patients are still receiving IVIG. Two patients were not considered in the analysis because of the withdrawal from the study for receiving HLA-identical BMT (Subject 8 and 17). Subject 1 was not on IVIG. At last F-U 11 out of 15 patients were off IVIG (**Table 1.4.6**).

Table 1.4.6 Requirement for IVIG Infusion

IVIG supplementation	N=15
Off IVIG at baseline	N 0
Stop during 0-3 yrs	N 6
Stop during LTFU	N 5
Off IVIG at last F-U	N 11

Abbreviations: IVIG=intravenous immunoglobulin; Y= Yes; N=No; LTFU=long term follow-up

a: Subject 1 (Pilot 1 Study) is excluded from this analysis as this subject's data regarding IVIG supplementation prior to Year 13 are not included in the clinical database.

These data about IVIG weaning during F-U, provide evidence for functional B cell and immunoglobulin production in the periphery. These data are also in line with the post-treatment decline in severe infections and vaccination responses.

- *Vaccination Responses*

Vaccine responses were evaluated for all subjects who received vaccination during follow-up, by measurement of antigen-specific responses.

IgG antibodies were detected at multiple time points following vaccinations against rubella, measles (rubeola), pertussis, tetanus, parotitis, and hepatitis B. Detectable antibodies to pertussis were found in 6 patients, to tetanus toxoid in 11

patients and to hepatitis B surface antigen in 8 patients. Moreover, two subjects received live attenuated measles-mumps-rubella vaccine and had detectable antibodies post-vaccination (Subject 5 and 6). The vaccinations were performed at least 3 months after IVIG discontinuation. Antibodies were generally detectable at multiple time points, indicating long-lived antibody production. Further, subject status updates obtained during LTFU reported on-time receipt of vaccinations for the majority of subjects in both the Pivotal population and supportive studies. Vaccine response data are in line with the post-treatment decline in severe infection rates and the decreasing use of IVIG in LTFU.

These data provide supporting evidence of humoral immune reconstitution, and B cell development and functionality, as well as overall lymphocyte functionality following gene therapy.

1.4.5.3 Autoimmunity

Autoimmunity was not assessed as an efficacy endpoint, but is related to T cell and B cell function and therefore relevant to assessment of immune reconstitution.

After gene therapy, 11 subjects reported a total of 26 events related to autoimmunity. Grade 1 ANA positivity was the most frequently reported treatment-emergent autoimmune event across subjects (in 7 patients at different time points, the majority in LTFU). The clinical relevance of these events is not clear as positive ANA titers without clinical signs of inflammatory or autoimmune conditions pose a minimal risk for such disease in the pediatric population (163, 164).

Four subjects had 6 SAEs of autoimmunity (anti-neutrophil antibody induced neutropenia, autoimmune thrombocytopenia [2 events], autoimmune aplastic anemia, autoimmune hepatitis, and Guillain-Barré syndrome). All autoimmune SAEs resolved and none were deemed related to the investigational treatment by the investigator. With one exception, all serious events occurred within the first 3 years following gene therapy.

Subject 7 had a family history of autoimmunity and had repeated autoimmune manifestations between approximately 2 years and 4.5 years following gene therapy, and received prednisone and three doses of PEG-ADA during the first event (autoimmune

hepatitis) in an attempt to restore immune function and reduce the observed autoimmunity, with the resolution of the event. The subject also developed a SAE of aplastic anaemia, probably of autoimmune origin, and non-serious AEs of autoimmune haemolytic anaemia and autoimmune hypothyroidism (3 years after gene therapy). The events responded to treatment with anti-CD20 monoclonal antibodies. The haemolytic anaemia resolved but the autoimmune hypothyroidism was reported as ongoing at the time of data cut-off.

Subject 8 had a history of autoimmune hemolytic anemia prior to gene therapy. Starting 4.5 months following gene therapy, this subject experienced repeated episodes of autoimmune thrombocytopenia. This subject had incomplete immune reconstitution and as a result, long term PEG-ADA was reintroduced starting in July 2006. The subject was considered to have an unsuccessful response to gene therapy and was withdrawn from the study in February 2008 when a sibling-matched BMT became available. Evaluations of autoimmune antibodies showed a diffuse positivity for anti-nuclear antibody, anti-neutrophil cytoplasmic antibody, anti-smooth muscle antibody, direct Coombs test, liver kidney microsomal antibody, and mitochondrial antibody.

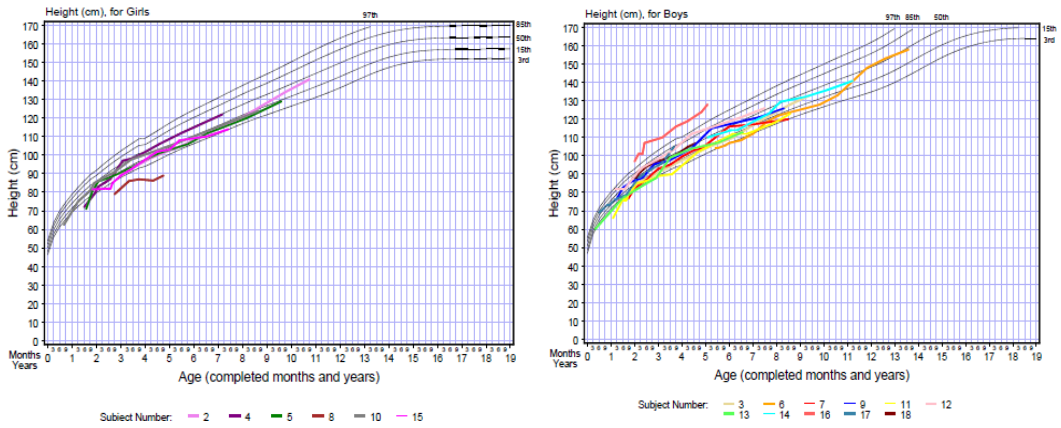
Subject 9 had an SAE of Guillain-Barré syndrome 3 years after gene therapy. The subject was hospitalised and underwent comprehensive biochemical, microbiological and virologic evaluation, including neuroimaging and nerve conduction studies. The tests confirmed Guillain-Barré syndrome, which was considered by the investigator to be not related to study treatment. The subject was treated with high dose IVIG and the event resolved.

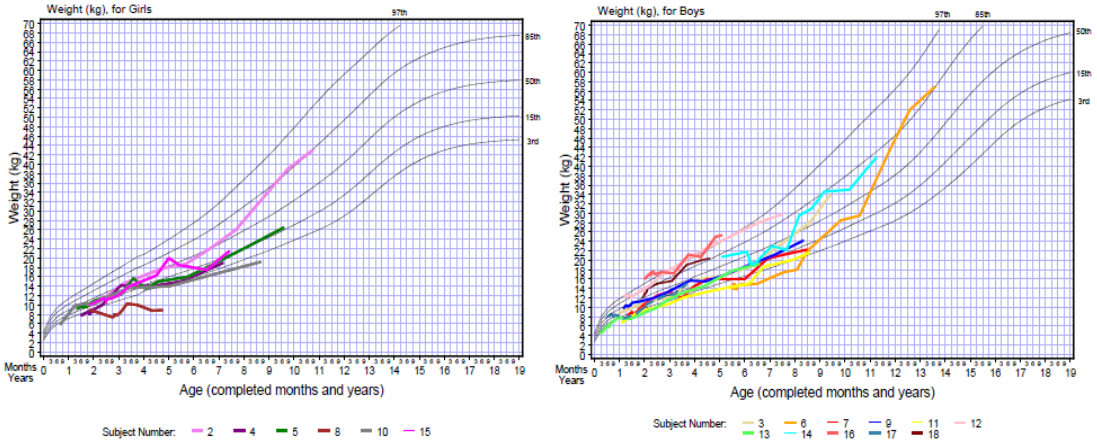
To note, Subject 13 had Grade 2 autoimmune hypothyroidism approximately 1 year after gene therapy, ongoing at the time of data cut-off.

1.4.6 Physical Growth

Individual growth profiles are shown for all subjects treated with gene therapy (**Figure 1.4.7**). Several subjects had height or weight that transiently fell below the curve at individual time points, but the majority either maintained or improved their age-appropriate height and weight relative to standard curves. However, Subject 8 had severe baseline disease activity and autoimmunity events considered as contributing factors to failure to thrive and Subject 10 was below the curve for weight only through Year 8, but was not far below the 5th percentile threshold.

Figure 1.4.7 Individual Subject Profiles of Height and Weight by Gender





1.4.7 Biological Activity

Biological activity was evaluated by means of measuring engraftment of gene modified cells (as determined by VCN) in bone marrow and peripheral blood cells (CD34+ stem cell, erythroid, granulocyte, and lymphocyte lineages), ADA activity in bone marrow and peripheral blood lymphocytes, and correction of the systemic metabolic defect as measured by dAXP levels in RBCs from bone marrow and peripheral blood.

1.4.7.1 Vector copy number

The ADA-transduced CD34+ cells and their progeny were isolated from purified bone marrow and peripheral blood through the last evaluation. Overall, there was a multilineage engraftment of ADA-transduced cells persisting over time (129).

We report the behavior of peripheral blood granulocytes, as an example of a cell lineage that is not normally impacted by ADA-SCID, and lymphocyte populations which are profoundly negatively impacted by ADA-SCID. CD15+ granulocyte marking in peripheral blood was typically 0.5 to 3% during follow-up (**Figure 1.4.8**). Gene modified cells appeared in the circulation in the first months following gene therapy, and the numbers remained stable for the duration of follow up (>5 years). As granulocytes do not have a survival advantage following gene transduction, the percentage of gene marked CD15+ granulocyte is a surrogate marker of the proportion

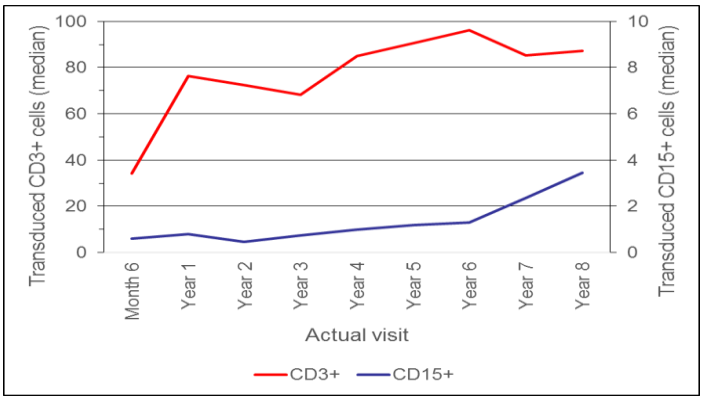
of gene modified stem cells in bone marrow. The proportion of gene modified CD34+ cells in bone marrow is consistent with the results for CD15+ cells in peripheral blood.

Bone marrow stem cells carrying the ADA transgene were able to support lymphocyte development in the periphery. Gene marking in CD3+ T cells was approximately 60 to 90% between Years 1 and 5 of follow-up (**Figure 1.4.8**).

These data show there was no loss of gene marking in engrafted bone marrow and peripheral cells over 8 years of follow-up after gene therapy.

Similarly to granulocytes, a relatively low proportion of gene modified cells was shown in the erythroid lineage, consistent with lack of survival advantage for this blood lineage.

Figure 1.4.8 Persistence of *ADA*-transduced cells in blood over time



An exploratory responder analysis was conducted to determine the proportion of subjects at each time point with bone marrow engraftment and peripheral circulation of gene modified cells. Both of the following criteria had to be met to be considered a responder:

1. Presence of ≥0.1% gene marked cells in bone marrow for both CD15+ granulocytes and erythroid cells (assessed by glycophorin A) and at least one of the CD3+ T cell, CD19+ B cell, or CD56+ NK subsets; and
2. Presence of ≥10% gene marked cells in peripheral blood CD3+ T cells and CD19+ B cells.

The definition of a responder was developed from previous experience with other gene therapy studies (165).

Among subjects with available data, 11 of 15 subjects (73%) were responders at Year 1. This trend was maintained throughout follow-up to Year 8, with 79% to 100% of subjects per time point considered as responders.

1.4.7.2 ADA Enzyme Activity

The presence of ADA was documented through detection of its enzymatic activity in blood mononuclear cells, marrow mononuclear cells, plasma, and red blood cells and was confirmed through flow cytometry of T and B cells, and monocytes.

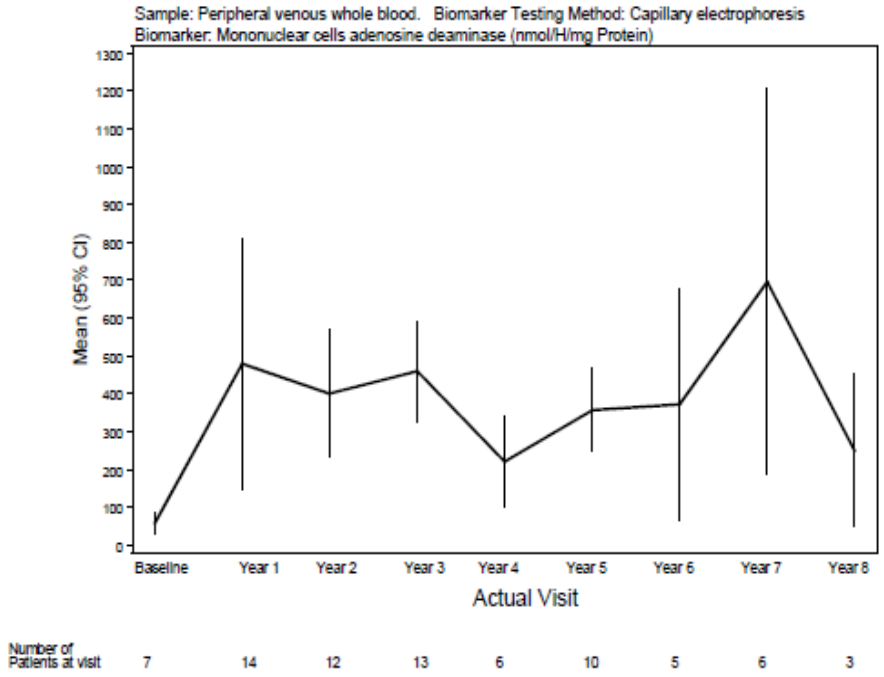
Plasma ADA activity by Year 1 showed increased levels in comparison to baseline, that were maintained for the duration of follow-up to Year 8.

All subjects apart from Subject 1, Subject 2 and Subject 3 were receiving PEG-ADA prior to gene therapy (**Table 1.4.1**). Per protocol, PEG-ADA was discontinued 11 to 22 days prior to gene therapy, and possible carryover effects of PEG-ADA exposure on early ADA activity could be present. Intracellular lymphocyte ADA activity was low at baseline, consistent with the disease. Following gene therapy, there was a marked increase in ADA activity in lymphocytes that was maintained for the duration of follow-up (**Figure 1.4.9**). Minor levels of ADA activity were evident in other cell lineages (e.g., erythrocytes), consistent with the relatively low levels of gene marking in these populations.

ADA activity ≥210 nmol/h/mg represents 10% of the mean value for healthy subjects and is considered clinically relevant as it is a threshold for minimum normal activity and corresponds to a minimum ADA activity level reported to result in normal function (56, 166).

Among subjects with available data, most had ADA > 210 from Year 2 onwards: 8 of 12 subjects (67%) met response criteria at Year 2. This trend was maintained throughout follow-up to Year 8, with 85% to 100% of subjects per time point considered as responders at each time point except Year 4, for which only 6 subjects had available data among whom 2 (33%) were responders.

Figure 1.4.9 Means (95% CI) of Lymphocyte ADA Activity



1.4.7.3 Purine Metabolites

Levels of dAXP were measured in RBCs from bone marrow and peripheral blood. There was no appreciable reduction in dAXP levels in RBCs in the first months following gene therapy, since baseline values were already low due to carryover effect of prior PEG-ADA use and the low number of mature cells in the periphery arising from gene-modified stem cells. Post-baseline mean and median dAXP were below pathological levels (100 nmol/mL) and this trend was maintained for the duration of follow-up. This was evidenced for dAXP levels in bone marrow-derived RBCs, with no decrease from baseline observed in the bone marrow. It was shown restored adenosine and adenosine metabolite clearance, evidenced in peripheral RBCs, by 6 months post-gene therapy (data not shown).

A responder analysis was conducted to determine the proportion of subjects at each time point assessed who had adequate systemic metabolite detoxification (dAXP in RBCs). A threshold of <100 nmol/mL dAXP in peripheral RBCs was chosen based on observations in patients treated with standard HLA-identical sibling donor SCT, was considered clinically meaningful and pre-specified for definition of responder (89, 128, 167-169). In addition, dAXP levels have been correlated with ADA-SCID disease

severity (56). In this way, correction of the systemic metabolic defect of ADA deficiency was demonstrated by analysis of bone marrow from Year 2, with 100% of subjects considered as responders in bone marrow analyses from Year 2 through Year 8. From analysis of peripheral blood, 92% of subjects were responders at Year 2, generally maintaining through Year 8 of follow-up.

1.4.8 Subjects with Unsuccessful Response to Gene Therapy

Poor engraftment of gene modified bone marrow cells led in some cases to lack of response to gene therapy treatment, poor immune reconstitution and inadequate correction of the systemic metabolic and consequent suboptimal clinical benefit. Subjects who received GT and then required post-gene therapy PEG-ADA for ≥3 months continuously or allogeneic transplant were excluded from efficacy analyses upon meeting either criterion and are also considered clinically to have experienced unsuccessful response to gene therapy.

Three subjects met these criteria:

- Subject 2 received two doses of gene therapy not in line with the recommended dosing and preconditioning regimen (first dose was below the recommended lower limit of cells and the second dose was without busulfan); this led to lack of engraftment. The persisting lymphopenia required reintroduction of short-term PEG-ADA for 2 months (from 2.4 years from the first gene therapy), before a new dose of gene therapy was administered. Long-term use of PEG-ADA was necessary from 4.5 years after the first gene therapy persisted during LTFU.
- Subject 8 presented with a history of hemolytic anaemia that required steroidal treatment before and during gene therapy. A SAE of autoimmune thrombocytopenia required restart of PEG-ADA 5 months after gene therapy. In this case, the poor engraftment led to continuous administration of PEG-ADA for all the duration of follow-up until withdrawal from the study following allogeneic transplant from a sibling donor that was not available at the time of study enrolment.

- Subject 17 restarted PEG-ADA 0.34 years after gene therapy. This subject received further PEG-ADA intermittently on additional occasions due to poor immune reconstitution, then continuously through the end of the 0-3 year follow-up period. This subject received a sibling donor allogeneic SCT and was withdrawn from the study.

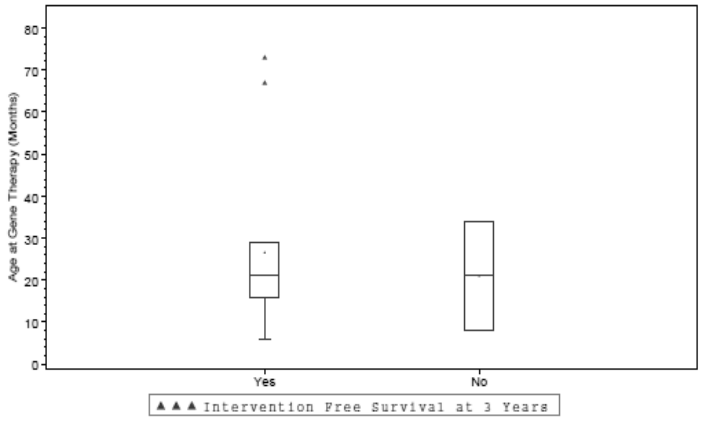
1.4.9 Baseline Predictors of Efficacy Post-Gene Therapy

Exploratory analysis was performed on the patient population to search for potential baseline predictors. Subjects with missing data or who started PEG-ADA were excluded.

These additional integrated analyses did not show any clear relationship between baseline covariates and efficacy outcomes. In addition to CD34+ cells/kg*VCN, the other baseline variables considered were age at gene therapy; CD34+ cells/kg; and baseline values for peripheral CD3+ T cell counts, TREC counts, peripheral RBC levels of dAXP, and BMI. The outcomes assessed were intervention-free survival (for the first 3 years following treatment), changes in T cell counts at Year 3, and changes in dAXP levels in RBCs at Year 3.

The lack of correlation between age at the time of gene therapy and these endpoints is demonstrated in a boxplot for intervention-free survival at 3 years (**1.4.10**), scatterplot for CD3+ T cell counts (**Figure 1.4.11**), and scatterplot for peripheral RBC dAXP levels (**Figure 1.4.12**).

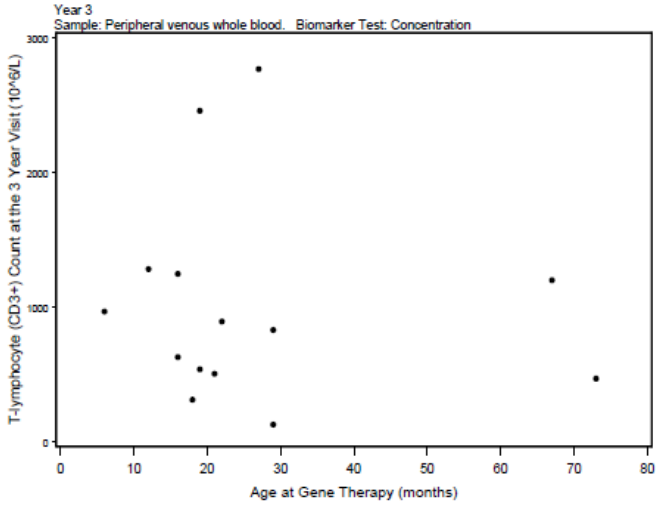
Figure 1.4.10 Boxplot of Intervention-Free Survival at 3 Years versus Age at Gene Therapy



Note: "Yes" indicates an intervention event occurred.

**Note (for Figures 1.4.9, 1.4.10, 1.4.11): Analysis includes N=14; Subject 1 did not have available data to contribute to 0-3 year data analyses other than survival and duration of follow-up, Subjects 8 and 17 data were excluded from the point that they began long-term PEG-ADA use, and Subject 18 had data available up to the Year 2.5 time point as of the data cut-off (08 May 2014).*

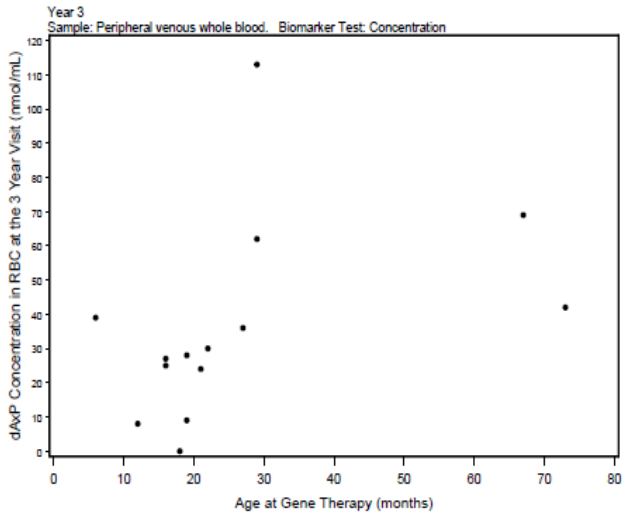
Figure 1.4.11 Scatterplot of Peripheral Blood T Cell Counts at 3 Years versus Age at Gene Therapy



Pearson Correlation Coefficient = -0.098

Note***(see Figure1.4.9)*

Figure 1.4.12 Scatterplot of dAXP Levels in Peripheral Blood RBCs at 3 Years versus Age at Gene Therapy



Pearson Correlation Coefficient = 0.421
Note**(see Figure1.4.9)*

1.4.10 CNS Manifestations of ADA-SCID

CNS abnormalities are frequent manifestations of ADA-SCID, even in patients that have been treated long-term with either ERT or SCT (81, 85).

Although the duration of follow up reported here is relatively short there is no evidence of benefit or modifications of CNS abnormalities after gene therapy and this suggest that the neurological findings and features are not reversed nor delayed by this treatment. In one analysis from literature about CNS outcomes, half of patients with prior ERT who received allogeneic hematopoietic SCT developed CNS abnormalities, despite adequate metabolic detoxification (i.e., dAXP levels) (85), giving the idea that CNS manifestations of ADA-SCID are not expected to improve following standard of care (conventional hematopoietic transplant). Toxic metabolite levels in patients receiving gene therapy have been shown to be comparable to those receiving SCT; however, no clear rationale for superior or inferior outcome with regard to CNS complications can be drawn from the available data.

The most frequently reported CNS events were cognitive disorders (5 subjects).

Two of the subjects considered to have unsuccessful gene therapy had CNS events.

The median time to onset of post-gene therapy CNS events for all subjects was 3.07 years (range: 0.95 to 13.26 years). Most events were reported as ongoing (15/22 events).

1.4.11 Quality of Life Evaluations

Non-standardized and informal pediatric quality of life assessments have been performed in LTFU by subject status updates at annual follow-up visits, investigating in attendance at school, participation in sports, eating habits, and receipt of childhood vaccinations on the recommended schedule. Although these assessments were not pre-specified as efficacy endpoints and baseline assessments were not collected, they provide some indication of the clinical benefit of gene therapy at LTFU time points with regard to overall well-being and daily function.

The majority of subjects across all studies who had available LTFU data have reported on-time vaccinations, attendance at school or pre-school as appropriate for the subject's age, and eating well with a varied and adequate diet. Most subjects have not reported participating in sports during the LTFU, primarily due to their parents' choice.

1.4.12 Safety of the infusion of medicinal product

The infusion of the medicinal product (autologous retroviral transduced CD34+ cells) was well tolerated by all patients and did not induce any adverse drug reactions in the hours following its administration.

1.4.13 Overall safety surveillance

1.4.13.1 Common Adverse Events (AEs)

The most frequently reported AEs were infections and infestations, blood and lymphatic system disorders, and skin and subcutaneous tissue disorders. All subjects reported Grade 1 AEs and 17 of 18 subject reported Grade 2 AEs.

The most frequently reported Grade 3 (severe) AE was device-related infection (5 subjects) and more than half of subjects reported Grade 3, infectious AEs. Other frequently reported (≥2 subjects) Grade 3 (severe) AEs were hepatic increased liver enzymes, anemia, neutropenia, gastroenteritis, pneumonia, and varicella. All of the most frequently reported Grade 3 AEs resolved.

Five subjects (28%) had Grade 4 events in the SOC of blood and lymphatic disorders.

Three of the Grade 3 neurologic/hearing AEs are ongoing (hearing impaired, moderate mental retardation, severe mental retardation).

1) Infections

Four subjects experienced varicella and all of these subjects recovered. Other less commonly reported opportunistic infections that occurred (and resolved) mostly out to the 3 year follow-up were experienced by 4 subjects: Aspergillus, cryptosporidial gastroenteritis, pulmonary mycosis and Cytomegalovirus infection in the same subject, Epstein-Barr virus infection. Oral candidiasis was reported in 6 subjects. Five of the 6 subjects that reported oral candidiasis post-treatment received multiple courses of antibiotics prior to the events, many as prophylaxis. Two of the 5 subjects had events pre-treatment (Subject 4 and Subject 5). The highest densities were from the pre-treatment to 3-month hospitalization period, prior to immune reconstitution. The 1 subject with an event during the 4-7 year follow-up (Subject 4) also had events pre-treatment and during the 3-month hospitalization period.

One subject had meningitis and three reported sepsis and all subsequently recovered. The latter three had CVCs in place, which is a known risk factor for sepsis.

Six subjects reported 10 CVC infections, half of which were classified as SAEs. Most of these events (8/10 events) were Grade 3 and developed in the 3 month hospitalization or 3-year follow-up phase.

A total of 12 subjects reported ear infections with onset mostly in the pre-treatment to the 3 year follow-up period.

Urinary tract infections were one of the most commonly reported infections. Overall, 10 subjects reported 21 UTIs, with no predominant organism reported. The type of UTIs reported (enterococcal, Escherichia coli, Pseudomonas, and Klebsiella UTIs) are the same as the organisms that cause UTIs in the general population. Most of

these events were reported during the 3-month hospitalization phase and early post-discharge and were not related to catheter placement. Seven subjects had recurrent UTIs: 5 subjects experienced 2 UTIs, and 2 subjects experienced 3 or more UTIs. Two subjects had serious UTIs. The incidence of UTIs in males (6 males vs. 4 females) is higher than expected in a general population of pediatric patients. Three of the male subjects had only 1 UTI, and 3 had 2 UTIs. Three of the male subjects who experienced a UTI had congenital abnormalities ongoing at Screening, that are known to cause a predisposition for UTIs (2 subjects had phimosis [Subjects 3 and 9] and 1 subject had cryptorchidism [Subject 13]).

2) Blood and lymphatic system disorders

The blood and lymphatic system disorders AEs reported in 5 or more subjects were anemia and neutropenia. Anemia, neutropenia, febrile neutropenia, bone marrow failure, and/or thrombocytopenia were reported in 12 subjects.

Five subjects (28%) had Grade 4 events and most occurred during the 3-month hospitalization phase. Five Grade 4 AEs were reported in the SOC of blood and lymphatic disorders (4 neutropenia AEs [1 of which was an SAE] and 1 thrombocytopenia AE). All of the Grade 4 blood and lymphatic disorder AEs occurred in the 3-month hospitalization phase and the majority occurred within 35 days of gene therapy, suggesting a correlation with chemotherapy with busulfan.

3) Hypersensitivity

Seventeen subjects reported 46 hypersensitivity events on or after treatment. The most frequently reported (≥3 subjects) events post-treatment were blood increased immunoglobulin E, atopic dermatitis, dermatitis, eosinophilia, and rash. Most subjects had events in the 3-year follow-up phase (n=12) and most had Grade 1 events. Dermatitis and rashes are common in children with ADASCID (170) and in healthy children. The median time to onset of hypersensitivity events was 1.46 years (range 0.02 to 11.84).

Skin papillomas were reported in 4 subjects (3 of 4 had single events of short duration) and all resolved. Skin papillomas are caused by the human papilloma virus

(HPV) and are considered benign tumors. They have been reported in late-onset ADA deficiency (171) as consequence of the defect in cellular immunity including T cell and natural killer (NK), that is crucial to host defense against HPV.

4) Hepatic Laboratory Abnormalities and Events

Seven subjects experienced adverse events of hepatic transaminase elevations between 2X and 5X ULN, nearly all in the treatment and 3 month hospitalization phases, and most were likely a result of busulfan conditioning, as they resolved.

Four subjects experienced hepatic steatosis (3 reported during LTFU) and 4 subjects had hepatomegaly (2 reported pre-treatment). Of the 4 subjects with hepatic steatosis, 1 also had cholelithiasis and dyslipidemia (Subject 3), 2 had increased hepatic enzymes (Subjects 2 and 6), and Subject 6 also had an AE of hepatic fibrosis, a SAE of Epstein-barr virus, and high exposure to busulfan. Other subjects with more than 2 hepatobiliary disorders or hepatic laboratory abnormalities included Subject 7 (hepatomegaly, hepatic enzymes increased, and autoimmune hepatitis) and Subject 14 (gallbladder cholestolosis liver disorder, hepatic lesion, and hyperplastic cholecystopathy).

Hepatic steatosis is associated with nucleotide turnover, loss of ATP and generation of adenosine monophosphate (AMP) and enzymes of the purine metabolism (AMPK, AMPD2) are involved in controlling fat accumulation in the liver (172). Although there are no published reports in the literature of hepatic steatosis being associated with ADA-SCID, Sokolic et al. reported at 2013 CIS meeting that 2 subjects on ERT and one after gene therapy presented with metabolic syndrome and fatty liver (173). Moreover, two ADA-SCID patients on long-term enzyme replacement therapy (who did not receive gene therapy), followed at San Raffaele Hospital showed fatty liver alterations (*Aiuti, personal communication*). In the paediatric population, hepatic steatosis is often seen in non-alcoholic fatty liver disease (NAFLD) which involves intrahepatic fat accumulation that could include various degrees of necrotic inflammation and fibrosis (non-alcoholic steatohepatitis). Paediatric NAFLD prevalence is estimated to be between 3% and 10% (174). The cause is unknown but risk factors include features of the metabolic syndrome, namely, obesity, hypertryglyceridemia, insulin resistance, and type 2 diabetes mellitus. Other than one event of dyslipidemia, no subjects had metabolic syndrome risks. Further investigation

on the predisposition to fatty liver in ADA SCID patients is needed, but this event is unlikely to be an ADR.

5) Oncogenesis events

No malignancies were reported. Subject 12, a male aged 1.3 years at gene therapy, was diagnosed with "two millimetric nodules in upper right pulmonary lobe", without symptoms, approximately 3 years after gene therapy. This subject also had an SAE of lipofibroma (175).

A total of 4 subjects reported AEs of Grade 1 electrophoresis protein abnormality, one subject pre-treatment (Subject 10), 1 subject during the 3-month hospitalization phase, and 2 subjects during the 3-month to 3-year hospitalization phase, all of which resolved (Subject 5, 6, 10 and 17).

Cytogenetic analyses, bone marrow morphology and bone marrow immunophenotype did not show major alterations. No abnormal blast values were found in either peripheral blood or bone marrow and therefore no AEs were reported.

1.4.13.2 Serious Adverse Events (SAEs)

Fifteen out of 18 subjects have reported 39 SAEs post-treatment. Twenty-two of the 39 SAEs reported post-treatment were infectious. Two subjects had Grade 4 SAEs infections (see paragraph 1.4.4).

The third Grade 4 SAE was a Grade 4 Neutropenia, that occurred in Subject 15 from Day 12, persisting to Day 45. The subject received antibacterial prophylaxis from Day 12. Two doses of G-CSF were administered on Days 38 and 42. The prolonged neutropenia was ascribed in part to conditioning related-myelotoxicity, and in part to an autoimmune phenomena, as anti-neutrophil antibodies were present. For this reason, the subject was treated with high dose IVIG. The event resolved after a duration of 29 days.

For autoimmunity SAEs see paragraph 1.4.5.3.

One subject had an SAE of hypertension (Subject 8) for which long-term anti-hypertensive therapy with amlodipine was administered.

1.4.14 Dose-Response Relationship

The number of CD34+ cells administered is considered a key parameter in the successful outcome in bone marrow transplantation.

In many transplant centers the minimum acceptable dose is considered to be $2x10^6$/kg CD34+ cells for a single transplant (176). Furthermore, recent published consensus guidelines for autologous hematopoietic SCT recommend a minimum dose of $2x10^6$ CD34+ cells/kg with a preferred target of $5x10^6$ CD34+ cells/kg (177). A maximum dose for CD34+ cells in autologous and allogeneic transplants is not typically defined, as the maximum dose often depends on practical and technical issues, such as the maximum number of bone marrow/cells that can be mobilized and collected from a single patient and, in case of major manipulation, the manufacturing process capacity.

Based on the above considerations, the dose of $2x10^6$ CD34+ cells/kg seems an appropriate lower dose limit supported by literature. Additional support for $2x10^6$ CD34+ cells/kg being a minimally effective dose comes from Subject 2 in Pilot Study 2, for whom a first dose of $0.9x10^6$ CD34+ cells/kg was not effective, leading to low gene marking and persisting lymphopenia, and the need of PEG-ADA administration. As already said, this subject also received gene therapy pre-conditioning with oral busulfan rather than via intravenously administration, and the product VCN for this patient was undetermined at the time of gene therapy, both factors that may have influenced the outcome of GT.

No formal dose ranging studies have been conducted, but the great variability in the number of CD34+ cells harvested among the treated patients and the variability in the capacity of cells' expansion during manufacturing, created a range of doses from 0.9 to $18.1x10^6$ CD34+ cells/kg into the GT development program, permitting an evaluation of safety and efficacy dose response across this range.

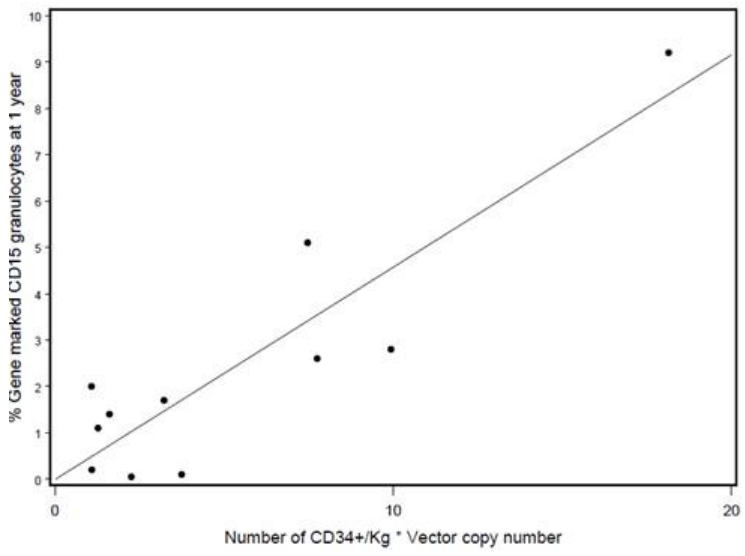
An exploratory analysis of dose response conducted on the Pivotal study population 0-3 year data suggested a correlation between the dose (defined as CD34+ cells/kg*VCN) and the efficacy outcomes, including correlations between dose and 1-year post dose granulocyte gene marking (R=0.8039) (**Figure 1.4.13).** Granulocyte gene marking is considered to be a good indicator of gene modified stem cell engraftment in bone marrow, so this is interpreted as evidence of higher doses of gene modified cells leading to increased engraftment of gene modified stem cells.

Further positive correlations were also observed between dose and Year 3 T cell count (R=0.5570), Year 1 lymphocyte ADA activity (R=0.6432), and Year 2 lymphocyte ADA activity (R=0.7567).

These data also suggest that the number of CD34+ cells that a patient can donate at baseline is a predictor of long term outcome. For this reason, it will be proposed for further development that patients should be able to donate a minimum of $4x10^6$ CD34+ cells per kg body weight to be eligible for gene therapy (this is the number of cells that should be available at the end of cell purification).

The dose response analysis was extended on the overall patients population dataset (excluding 2 subjects who required PEG-ADA reintroduction within 6 months post-gene therapy) to assess correlation between dose and intervention-free survival during 0-3 years, change from baseline in T cell count at Year 3, and dAXP levels in RBCs at Year 3. No correlation between dose and clinical response was shown.

Figure 1.4.13 Gene Therapy Dose (CD34+ cells/kg*VCN) versus Percentage of Gene Marked CD15+ Granulocytes at 1 Year (Pivotal Population)



In the main clinical trial, a maximum of $40x10^6$ CD34+ cells/kg were to be taken from a subject's bone marrow and the maximum recommended reinfusion dose was $20x10^6$ CD34+ cells.

There have been no dose-limiting toxicities observed within the administered dose range of $0.9x10^6$ to $18.1x\ 10^6$ CD34+ cells/kg. An upper limit for the medicinal product dose has been defined based on practical and manufacturing limitations.

1.5 DISCUSSION

1.5.1 Efficacy of Gene Therapy with autologous transduced CD34+ cells for the treatment of ADA SCID patients.

This thesis reports clinical data from 18 ADA SCID subjects treated with autologous CD34+ cells transduced with retroviral vector encoding ADA.

For all 18 subjects, prior PEG-ADA treatment had been ineffective or it was not a treatment option due to intolerance, lack of availability, or contraindication, andsubjects did not have a suitable sibling donor for HLA-matched SCT. Four subjects had received prior unsuccessful allogeneic SCT, and 15 subjects had previously received prior PEG-ADA. This characteristics highlight the high unmet medical need in this subject population.

Gene therapy was preceded by reduced intensity conditioning regimen. Preconditioning supported persistent engraftment of gene modified cells, expressing ADA-gene and resulting in correction of the ADA metabolic defect.

A 100% survival rate, that was a primary endpoint of the main clinical study, was observed for all 18 subjects treated with a median follow-up duration of approximately 7 years (up to a maximum of 13 years for one subject). This exceeds the 67% survival rate observed for MUDs recipients after a median follow-up of 6 years in conventional transplant settings (58).

Severe infection rates, one of the key secondary endpoints for the main study, were significantly reduced after gene therapy in comparison to baseline, and from Year 1 onwards remained lower than baseline, showing to further reduce in each year after gene therapy.

Evidence of immune reconstitution was observed from 6 months post-gene therapy, with significant increases in numbers of peripheral CD3+ T cells (another key secondary endpoint for the main study) and T cell subsets.

The increases in T cell subsets were accompanied by evidence of thymopoiesis (TRECs) and peripheral T cell function (i.e., robust proliferation responses to anti-CD3 antibody or PHA stimulation) from Year 1 onwards; no clear changes in CD16+ CD56+ NK cell counts were visible and B cell counts post-gene therapy were variable, with some increases and some decreases from baseline, but adequate function was

demonstrated by vaccine responses, increases in serum immunoglobulin production, and decreased IVIG requirement over time (especially from Year 4 onwards in LTFU).

Persistent evidence for engraftment of gene modified cells in multiple lineages in the BM and PB was observed, without loss of gene marking over 8 years of follow-up. Higher levels of gene marking in mature lymphoid cells supported the hypothesis that gene modified cells have a selective advantage.

ADA-gene expression, measured as ADA activity in bone marrow and blood, decreased during the few weeks after treatment, in line with discontinuation of PEG-ADA treatment, and then increased from around 1 year after treatment in both bone marrow and blood, after which expression was stable for the duration of follow-up, reflecting synthesis of ADA in transduced cells.

Peripheral blood lymphocyte ADA activity levels increased after treatment and remained stable during the follow-up, resulting in correction of the ADA metabolic defect measured by dAXP levels in RBCs.

The majority of subjects (15 out of 18) did not require PEG-ADA after gene therapy; 3 subjects experienced gene therapy failure and required long term post-gene therapy PEG-ADA (≥3 months continuous use).

Analyses of potential baseline predictors of benefit did not suggests any specific predictor for unsuccessful response to gene therapy. On this series experience, the availability of a minimum dose of $4x10^6$ CD34+ cells per kg body weight (after cell purification) at baseline has been set as an eligibility requirement for gene therapy and as a likely predictor of long term outcome. The absence of any correlation of outcome versus age supports an indication for gene therapy that covers a broad age range of 0-18 years.

There were no clear effects of gene therapy on CNS manifestations of the ADA SCID, as when CNS abnormalities were present prior to GT, these were not mitigated by the treatment, and as some patients developed CNS abnormalities after receiving gene therapy, similar to CNS outcomes in standard of care SCT of ADA-SCID (85).

Overall quality of life for subjects who have received gene therapy suggests these children are growing with their peers (from physical growth data on height and weight), are entering and maintaining regular school attendance, despite of eventual learning disabilities, and are participating in physical activities such as sports. This is a qualitative outcome that matches with the quantitative evidence of long-term clinical benefit.

1.5.2 Safety of treatment

No treatment-related mortality (TRM) was registered in the clinical program. Preparatory conditioning with busulfan was well tolerated by all subjects. Hepatic enzyme increases, anaemia, and neutropenia occurred mostly in the 3 month hospitalization period after GT and were considered to be related to busulfan conditioning. AEs of cytopenias, elevations in transaminases and hypertension, observed post conditioning and likely related to busulfan, generally resolved over time.

The infusion of the investigational medicinal product was well tolerated

No fatal AEs following treatment were reported; on the other hand, as expected in ADA SCID (72), all 18 subjects experienced infectious AEs. The 3 most frequently reported infectious AEs were normal, expected childhood infections, as upper respiratory tract infection, gastroenteritis, and rhinitis (178). Serious opportunistic infections were not common. The majority of severe infections occurred during immune reconstitution and, moreover, were often related to external factors, such as the presence of CVC or congenital abnormalities in several male subjects that experienced UTIs. This is particularly meaningful, as the majority of subjects (82%) experienced severe infections and on multiple occurrences in the pre-treatment phase, despite the incidence of pre-treatment events may be underreported.

No events indicative of leukemic transformation or myelodysplasia were reported.

No AEs consistent with systemic allergic events were identified within the first year after gene therapy administration.

Neurological events, including the cognitive and audiological events, were observed and are similar to those observed in patients treated with BMT or PEG ADA (89, 170). These events may be directly related to ADA SCID, to infections that subjects may have experienced (e.g., meningitis), or to other medications received (e.g., antibiotics such as gentamycin).

Overall, 10 subjects reported a total of 21 events related to autoimmunity with isolated antinuclear antibody positive as the most frequently and often nonspecific reported event. Three subjects had SAEs of autoimmunity and two of these subjects required reintroduction of PEG-ADA in order to reduce the autoimmunity phenomena. Autoimmune events in SCID patients are well described as a consequence of immune

dysregulation treated with HCT or PEG ADA during immune reconstitution (44) but may also be due to immune reconstitution that follows gene therapy.

1.5.3 Comparison of retroviral gene therapy with HSCT

HLA-matched sibling donor SCT is considered the gold standard in ADA-SCID therapy, but availability of a suitable sibling donor is limiting, making MUD transplant the more readily available and commonly used standard of care (10).

The outcome of allogeneic HCT transplantations for children with SCID has improved dramatically over time (81, 115), but an emerging awareness is developing that some forms of SCID are more difficult to treat than others, in particular patients with T-B- SCID do worse than T-B+ SCID. This is either more true for SCID forms that are caused by defects of molecules more ubiquitously expressed than restricted to the immune system only (58).

In particular for ADA SCID, the need for conditioning and the metabolic issues of the disease make transplantation from MMUD and HAPLO still characterized by increased risk of toxicity and poor outcome (58, 81, 82).

Therefore, improvement in morbidity and mortality over the existing therapeutic options remains mandatory and gene therapy is a valid option with a favourable benefit/risk in relation to MUD BMT.

Gene therapy is also potentially a treatment option for all patients without the need for a protracted donor search.

1.5.3.1 Overall survival

The results from *Hassan et al.* report documented, by retrospective analysis of transplantation among 106 patients with ADA-SCID, the different outcomes from different donor sources and after different conditioning regimens. They show clearly the importance of donor matching in improving outcome and the poor outcome in mismatched transplantations, as the most important factor influencing the success of the transplant. Overall survival was higher in genoidentical donor transplants (86% for MSD and 83% for MFD, both conditioned and unconditioned) than in HLA-matched MUDs (67%). Long-term survival rates of 67%, 43% and 29% were observed after

matched unrelated, haploidentical or mismatched unrelated donor respectively (58). Cumulative data from unrelated UCB transplant in primary immunodeficiencies show an estimate 5 year survival of 57% +/- 6% (81).

The objective of the primary efficacy analysis in the main gene therapy study was to compare survival with the historical MUD rates, and the 100% survival of all gene therapy recipients compares favourably with lower 67% OS of MUDs recipients (58). Importantly, the observed 100% survival of all subjects in the gene therapy program is also greater than the sibling donor survival rates reported. Moreover, even considering patients requiring intervention (>=3 months PEG-ADA or SCT) as treatment failures, the survival rate without intervention in pivotal population (92%) exceeds the overall survival rate for MUD transplants and sibling donor transplants, while the survival rate without intervention in integrated population (82%) remains higher than the MUD and is similar to the sibling donor survival rate.

The superior results after MSD/MFD HCTs may also reflect a shorter donor search to transplantation time, due to the possibility (because of the ready availability of the donor) to perform MSD/MFD HCTs without conditioning and soon after the diagnosis in a patient less compromised following multiple infectious events or ongoing metaboli degeneration, in comparison with unrelated donor transplantations, where the delay may affect negatively on the clinical status of the patient (58). In this view, an advantage of autologous gene therapy procedure is that it is soon available for all patients (that can donate adequate CD34 cells), and does not require a delay in treatment while a matched donor is located. Moreover, treatment with gene therapy does not preclude alternatives in patients who fail to achieve adequate immune reconstitution (i.e. PEG-ADA or SCT).

Age is indeed a significant risk factor in transplantation setting for ADA SCID patients, with a reported trend to better survival, even if not statistically significant, in children up to 6 months of age (58). Importantly, the median age at treatment was 1.7 years (range, 0.5, 6.1), higher than that reported by Hassan, being 4 months (range, 2 weeks to 7 years) (58). Moreover, the median available follow-up after gene therapy is 6.94 years (range: 2.6 to 13.4), comparable to the median follow-up of 6.5 years (range, 1.6-27.6) reported in Hassan cohort (58). These observations strengthen the better outcome of gene therapy if compared to MUD SCT as a treatment for ADA-SCID.

1.5.3.2 Conditioning regimens, complications and deaths

The majority of deaths (63%) after all types of transplantations for ADA SCID are reported in the first 100 days after HCT and are mainly related to severe infections, as pneumonitis/respiratory failure and sepsis, followed by GvHD (15%) and fungal infections (11%). Only 13 deaths occurred after 100 days and only 2 of them were related to transplant-related complications, namely chronic GvHD (58).

The use of myeloablative conditioning regimen in MUD/MMUD/HAPLO transplantations is an important factor playing a major role in determining the unfavourable outcome in ADA SCID patients, if compared to the unconditioned transplants from MSD/MFD. In particular, the absence of serotherapy in the MSD/MFD transplants allows a faster and better recovery of CD3+ cells in the first year after transplantation and in a decreased mortality for viral infections due to the more rapid T-mediated resolution of the events. Conversely, transplantations after RIC had an OS of 67%, and this was not significantly different from outcomes after unconditioned procedures (58).

As reported above, the use of non-myeloablative conditioning regimen with Busulfan 2 mg/kg/day for 2 consecutive days prior to GSK2696273 gene therapy was well tolerated and led to successful treatment of the disease. No fatal AEs following GSK2696273 treatment were reported. Also after gene therapy, complications, previously described as AE/SAE, were mainly infectious and were observed in all 18 subjects, highest in the pre-treatment to 3-month hospitalization period and then decreasing with time, a finding which would be expected in subjects becoming immune reconstituted. The most frequently reported infectious AEs were normal, expected childhood infections. Serious opportunistic infections were not common with only 1 subject each reporting *Aspergillus* infection, cryptosporidial gastroenteritis, and pulmonary mycosis. The majority of severe infections (12 of 15) were reported during the 3-month to 3-year treatment period and all resolved. Moreover, all the subjects experienced a reduction in the rate of infections after gene therapy, demonstrating the significant benefit of this approach. Other AEs thought to be related to busulfan were 4 episodes of neutropenia (1 of which was a SAE) and 1 episode of thrombocytopenia, occurring during the first 35 days from gene therapy. Moreover, 7 subjects showed a moderate increase of liver enzymes, as expected after chemotherapy, without other signs of liver failure. All the events resolved.

On the other hand, busulfan pre-conditioning was shown to be essential to obtain a good engraftment of engineered stem cells. Subject 2 received a first gene therapy after receiving oral busulfan; the CD34+cells dose was very low (0.9×10^6/kg), due to a low numbers of CD34 stem and early progenitor cells in the harvested bone marrow. The patient has persistet lymphopenia and a second harvest was performed, with a second infusion of CD34+cells at the dose of 2.1×10^6/kg 2.6 yrs after the first gene therapy, but without busulfan conditioning. The immune reconstitution was not complete and the patient remained on long term PEG-ADA since 4.5 yrs after the first gene therapy.

1.5.3.3 Engraftment, immune and metabolic reconstitution and outcome

While superior survival was seen in patients who received unconditioned transplants in comparison to myeloablative procedures (81 and 54%), unconditioned HAPLO T-cell depleted transplantations had a high rate of graft failure or rejection, differently from the data of similar transplantations performed in SCID-X1 (179). At a less degree, non-engraftment occurred also in genoidentical transplants (in 13% of unconditioned sibling transplants), despite overall it was reported an excellent outcome with stable high levels of T donor chimerism and excellent T-cell numbers and function (58).

Due to the lack of T and NK cells in ADA SCID patients, the basis of non-engraftment is unlikely to relate to immunologic rejection. An alternative explanation could be the inability of the ADA-deficient marrow stromal microenvironment to support engraftment of wild-type HSCs, as supported by in vitro murine data, while in case of infusion of whole marrow, as for MSD/MFD transplantations, even if unconditioned, the engraftment is mainly of more mature cells (58). In the HAPLO and MMUD setting, conditioned transplantations also have poor outcome, probably for the high toxicity of the regimen or the delayed T-cell reconstitution, with consequent inability to clear viral infections (58).

All subjects who received gene therapy showed evidence of engraftment of gene modified bone marrow and peripheral cells, and the majority (15/18 subjects) were considered treatment successes on the basis of immune reconstitution and evidence of therapeutic mechanism of action. Three subjects overall required intervention with

long-term PEG-ADA, and in two cases subsequent allogeneic SCT, and this intervention may have contributed to the 100% survival rate.

One of the most important advantages of gene therapy contributing to the benefits and the success of this approach, is the use of autologous cells in the treatment dose, which reduces the risk of graft versus host disease and rejection (55). This is a considerable utility, especially versus mismatched HSCT in which the risk of GvHD is considerable. The absence of risk of GvHD permits also to avoid the long-term administration of immune suppressive prophylaxes and/or treatment, usually favouring viral reactivations that represent a quite common complication following HSCT and can be severe. In our study, 4 patients experienced Varicella infection (3 in the 3 months-3 years follow-up and 1 at 6 year follow-up), 1 subject presented with pulmonary mycosis and consensual Cytomegalovirus infection and another subject underwent Epstein-Barr reactivation with persisting lymphopenia, both during the 3-month hospitalization phase. The EBV reactivation was a SAE which required back-up bone marrow cells on 2 occasions, at approximately 1 and 2 months after gene therapy. All these AEs/SAE were well controlled thanks to careful viremia monitoring and prompt start of specific therapy. No EBV-associated PTLD have been observed as of last available follow-up.

Long-term immune recovery in the *Hassan* cohort showed that regardless of transplant type, overall T-cell numbers were similar although a faster rate of T-cell recovery was observed following matched sibling or matched unrelated BMT. Post-transplant TREC levels were low in unconditioned transplants, suggesting more limited stem cell engraftment with this approach. Interestingly, humoral immunity and donor B cell engraftment was achieved in nearly all evaluable surviving patients, including 10/12 unconditioned patients, and most patients were able to discontinue immunoglobulin replacement. This supports the hypothesis from new B-cell reconstitution data in T-cell depleted haploidentical unconditioned transplants, that the purine salvage pathway is not as critical for B cells as it is for T cells, and that in ADA-SCID, host B-cell function can be reconstituted following donor T-cell reconstitution (58, 180). Another hypothesis, is that may also be a survival advantage to ADA-expressing B-lymphocytes (58).

According to the available data, the immunological and metabolic recovery after allogeneic transplant is well maintained even after 10 years or longer in some patients (81). Nevertheless delayed or suboptimal immune reconstitution as a result of poor early

engraftment or gradual decline in immune functions is observed in a significant fraction of surviving patients (81).

For patients who received gene therapy considered as treatment responders (15/18), the cumulative evidence suggested development and normal function of T cells including increases in peripheral cell counts and proliferation from approximately 6 months post-gene therapy and onwards, with the increased numbers and functionality of cells maintained from Year 1 through 8 years of follow-up. Recovery and maintenance of T cell counts over time was particularly significant and clearly sustained overtime, as demonstrated for subjects in the LTFU who remain alive and free of opportunistic and severe infections. Importantly, TRECs in peripheral blood lymphocytes were increased from Years 1 to 3 post-treatment, and gradually declined at Years 5 and 8, in line with age-related decreases in thymus size; this was in line with the increase in lymphocytes counts and the detection of peripheral CD4+ CD45RA+ naïve T cells. As this appears to be a stronger evidence of prethymic progenitor cell engraftment than happens in allogeneic transplant setting, it is very encouraging in expecting a long term immunologic recovery after gene therapy, being anyway a long follow-up mandatory either in patients undergone allogeneic transplant or gene therapy. Peripheral B cells counts did not show a clear increase post-gene therapy, but B cell function was evidenced by observed immunoglobulin production, vaccination responses, and decreased dependence on IVIG use over time. This evidence supports the possibility of one or both of the two hypothesis made before for B cells reconstitution. The biological evidence correlates with the timing of decreasing severe infection rates, which dropped post-gene therapy from Year 1 onwards.

In the Hassan cohort, a metabolic correction was evident in all the transplanted patients irrespectively from the donor source and the intensity of conditioning regimen (MAC/RIC/unconditioned) (58). The available data about long-term F-U after GT show comparable efficacy profile. The presence of gene modified bone marrow and peripheral cells across cell types (granulocytes, erythroid cells, T cells, B cells, and NK cells), elevated ADA activity, and reduced dAXP levels after receiving gene therapy collectively demonstrated the restoration of ADA function and correction of the metabolic defect and matched the timing of immune cell increases in numbers and function.

1.5.4 Comparison of retroviral gene therapy with ERT

The successful data reported from transplantations from a well-matched donor source show that, once patients survive the procedure and engraft donor cells, relatively complete immune reconstitution is achieved.

This observation is in striking contrast to the results of immune reconstitution after ERT. Evidence suggest that, despite being well detoxified, patients on long-term PEG-ADA present with T-cell numbers that are much below normal levels (58). The specific reasons are unclear, but the data suggest as the main reason that intracellular ADA expression either through transplantation of wild-type cells or GT of autologous cells corrects T-cell function more effectively than exogenous enzyme replacement. Moreover, differently from GT and BMT, the B-cell function defects are not fully repaired by ERT whereby only 50% of patients are able to discontinue Ig replacement therapy (58).

In the main study, the medicinal product was studied in patients for whom PEG-ADA was not a valid treatment option. Positive outcomes have been demonstrated in patients treated with gene therapy that had not received prior PEG-ADA with no difference in the outcome from those who had received prior PEG-ADA (109).

Although no comparative study between GT and PEG-ADA has been conducted, comparison between the data with GT and published data with PEG-ADA indicated a positive benefit risk for GT, particularly in relation to the 100% survival rate, and 82% intervention free survival rate following treatment with gene therapy and the durability of the immune reconstitution.

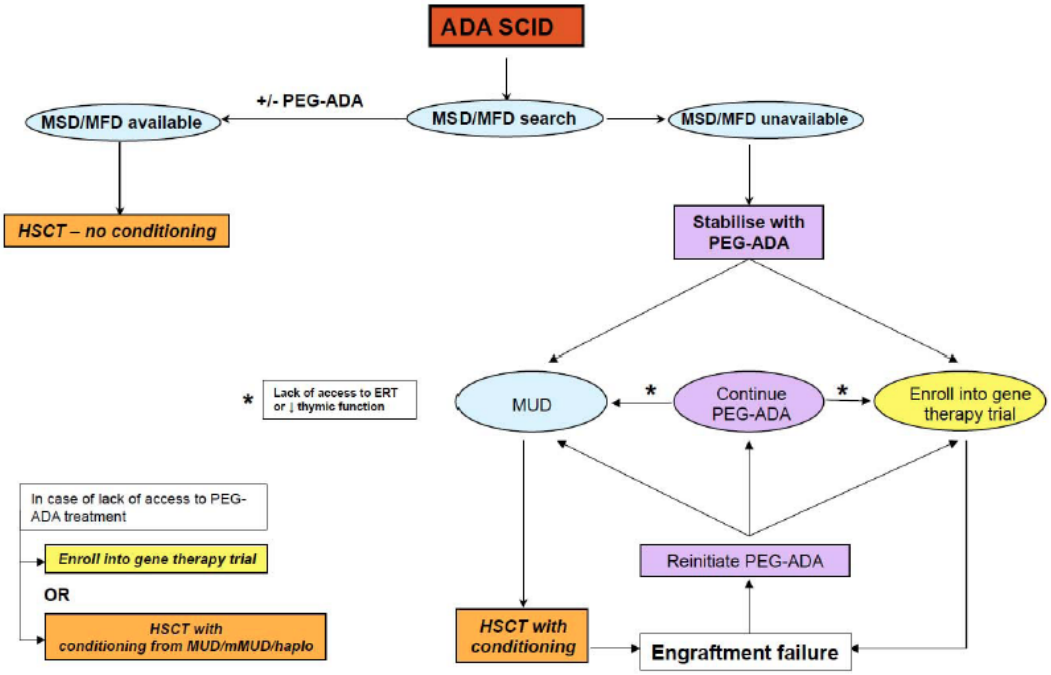
Given these data, the prevailing current medical opinion is that ADA-SCID patients should receive definitive treatment such as SCT or gene therapy at an early age because better outcomes are achieved with earlier treatment. The results from gene therapy trials led to issue recommendation from the EBMT Inborn Error Working Party, according to which gene therapy is considered a valid option to all patients without an HLA-identical sibling donor, regardless of the age, availability of a MUD, and outcome of PEG-ADA therapy (181).

A recent review by Gaspar (81) provides the policy at GOSH, which is aimed at first stabilizing patients with PEG-ADA and then offering patients MUD transplant or

gene therapy (**Figure 1.6.1**).

The ADA SCID population candidate to receive gene therapy in the next future will be extended to all ADA SCID patients aged 0-18 years lacking a MSD. The indication will be to perform the gene therapy treatment as early as possible, also in patients who show a good immunological response to PEG-ADA, without waiting the ERT failure.

Figure 1.5.1 From Gaspar et al. 2009, How I treat SCID.



1.5.5 Autoimmunity

Autoimmunity is occurring very often in SCID patients who achieve partial or poor immune reconstitution after conventional SCT or with PEG-ADA treatment (44, 182), and in late onset ADA deficiency (66, 67), with various mostly hematological and endocrine manifestations (44, 81, 108). It has been argued that when patients with early-onset ADA-SCID shift from a virtually complete lack of T-cell–mediated immune response prior to treatment to an intermediate level of T-cell function, this allows for the

development of allergy responses and autoimmune disease (44). This can happen following treatment with BMT, PEG-ADA, or also gene therapy.

After gene therapy, 11 subjects reported a total of 26 events related to autoimmunity. Grade 1 ANA positivity was the most frequently reported treatment-emergent autoimmune event (4 events), whose clinical relevance is not clear as positive ANA titers without clinical signs of without inflammatory or autoimmune conditions pose a minimal risk for such disease in the pediatric population (163, 164). Several subjects treated with GT entered their study with a history of autoimmunity and experienced subsequent post-gene therapy autoimmune events considered as serious. Two subjects who had post-gene therapy autoimmune events reported as SAEs had a history of autoimmunity, and 2 subjects who did not present at baseline with a history of autoimmunity subsequently developed autoimmune SAEs after gene therapy. Five out of 6 SAEs of autoimmunity (anti-neutrophil antibody induced neutropenia, autoimmune thrombocytopenia [2 events], autoimmune aplastic anemia, autoimmune hepatitis, and Guillain-Barré syndrome) occurred within the first 3 years following gene therapy. Two subjects (one of which was Subject 8 who had an unsuccessful response to GSK2696273 treatment) required reintroduction of PEG-ADA in an attempt to restore immune function and reduce the observed autoimmunity. Subject 8 had a history of autoimmune hemolytic anemia prior to gene therapy, that included chronic treatment with steroids, and had PEG-ADA reintroduced approximately 5 months after gene therapy due to an SAE of autoimmune thrombocytopenia, as already described (109).

Importantly, chronic autoimmunity by the time of gene therapy treatment might have contributed to an unfavorable environment to achieve successful gene therapy and consequent failure of engraftment in this subject. Another hypothesis is the implication of mutation of single genetic loci leading to susceptibility to multiple autoimmune diseases in some patients, presumably modifying a generalized predisposition to autoimmunity with organ/disease specificity, as postulated (44).

It remains unclear to what extent specific mechanisms of tolerance are affected in ADA deficiency (44). Reconstitution of effector T- and B-cells as well as metabolic detoxification after treatment might therefore be requirements for the onset of autoimmunity (44).

1.5.6 Safety considerations

1.5.6.1 Risk of Mutagenesis

Adenosine Deaminase is a house keeping gene, it is not involved in promoting cell proliferation (unlike Wiskott Aldrich Syndrome Protein or Rag 2 gamma in X-SCID) and is only expressed at relatively low levels following gene therapy.

In several gene therapy trials including the present one, where over 40 ADA-SCID patients have been treated since 2000, no leukemic or oncogenic events were revealed, even if integration into known proto-oncogenes have been reported for all the trials (55). In this series of ADA SCID patients treated with GT, no adverse events indicative of leukemic transformation, lymphoproliferation, or myelodysplasia have been reported. Moreover, analysis of bone marrow and peripheral blood smears has not revealed the presence of abnormal immature myeloblasts and no karyotypic alterations were found by cytogenetic analyses. In addition, serum protein electrophoresis revealed no serum monoclonal protein during long-term follow up, suggesting that the gamma globulins present were polyclonal, indicative of a normal B cell compartment.

It will be important to continue to monitor GT treated patients to ensure that existing data trends supporting the safety of gene therapy remain over the long term, but at this time, the benefit/risk seems very well supported in terms of leukaemia risk.

1.5.6.2 Risks Associated with Posology and Manufacturing

The recommended dose for GT (2 to $20x10^6$ CD34+ cells/kg) was determined by observations from hematopoietic transplantation practice, the GT clinical program, and constraints of bone marrow harvest and the manufacturing process.

Although no formal dose-ranging studies have been conducted, from the outcome gained from the administration of very different cell dose (between 0.9 and $18.1x10^6$ CD34+ cells/kg), a minimum recommended dose of $2x10^6$ CD34+ cells/kg would be suggested; this is also in line with pediatric SCT dosing literature (183). As approximately 10% of patients may not yield the minimum number (4×10^6) of CD34+ cells/kg required to ensure that the minimum dose can be manufactured, primarily due to the ADA deficiency in itself, an alternative is the use of a product derived from

frozen CD34+ cells in addition to the fresh product, to ensure manufacture and the infusion of an adequate quantity of CD34+ cells; this option is under evaluation.

Moreover, since cells are infused fresh under the current process, there is also the risk that, due to initial contamination of the bone marrow harvest, the final product would be compromised. In this case, the patient will not be infused and will receive the bone marrow back-up.

1.5.7 Benefit/Risk across the age of treatment

Although the actual age range of patients that received treatment ranged between 6 months to 6 years 1 month, there is a reasonable expectations that age range should be from birth to 18 years, as outcomes have shown no significant trend in relation to the age of patients treated to date in the gene therapy clinical program.

In the series of ADA SCID European patients undergone BMT reported by Hassan, only one patient out of 106 was older than five years old (58) and in a similar US study across all SCIDs reporting two hundred and forty patients none were older than five years of age (10). Occasionally patients that have only partially responded to previous treatments may present and treatment centres can consider all treatment options.

Although no information are available to support evaluation of GT in patients below six months of age, there are no data or hypotheses suggesting that GT would behave differently in very young infants and GT has not demonstrated age-related safety concerns. Very young infants may experience additional potential benefits of early treatment with GT due to more active thymopoiesis and generally reduced concomitant disease at the time of treatment.

With the increasing development of newborn screening (12) and well organised bone marrow donor registries, patients may present for treatment increasingly quickly. However, considering the time required to confirm ADA-SCID diagnosis, for patient's stabilization and preparation for the treatment, more frequent is going to be the situation in which patients > 6 months of age will be referred to treating centers. Moreover, the treatment of children aged 0-6 months presents particular challenges, as the technical difficulty of bone marrow harvesting, the limited volume of bone marrow that can be extracted and the increased variability of busulphan pharmacokinetics (in infants <9kg),

which may ultimately determine the age at which a patient may be safely undergo the treatment.

1.6 CONCLUSIONS

Gene therapy for ADA SCID has led to 100% long-term survival (maximum and median follow-up of 13 and 7 years, respectively). The majority of subjects have demonstrated evidence of multilineage engraftment of gene modified cells, sustained increases in functional gene modified lymphocytes, maintenance of a robust immune reconstitution, significantly fewer severe infections over time, and continued physical growth.

Overall, safety data show that the gene therapy program was as expected for a pediatric autologous HSCT. The frequency and severity of infections, severe, opportunistic and otherwise, trended downward as subjects became immune reconstituted. No events indicative of leukaemic transformation or myelodysplasia were reported and no issues around immunogenicity were evident. At this time, identified adverse drug reactions are limited to those thought to be related to immune reconstitution (fever and various autoimmune events).

Overall, based on the positive benefit to risk profile, the limitations associated with current therapeutic treatment options, and the significant mortality experienced by patients with ADA-SCID, there is an urgent medical need for additional therapeutic options. The gene therapy program developed at TIGET was inlicensed by GSK and is currently under further development, with the aim to obtain the registration in the market.

There is a robust evidence of efficacy of the data for an ultra-rare disease, considered fatal in the first year of life when untreated, and appropriate comparisons with other treatments.

1.7 FUTURE PLANS

Gene therapy has made significant progress over the last 20 years. Previous trials focused on the treatment of PIDs, and among them ADA SCID, with retroviral vectors have demonstrated clinical and biological efficacy. Unfortunately, in some cases of PIDs safety issues occurred, as hematological malignancies in 5/20 patients affected by SCID-X1, 3/5 patients treated for CGD and 7/10 patients affected by WAS (55).

Despite this, no leukemic or oncogenic events after the use of retroviral vectors for the treatment of patients with ADA SCID have been reported in over 40 patients treated in UK, US and Italy (138).

Although no vector-related harmful effects have been observed in any of the ADA-SCID trials, the development of new vectors with reduced risk of insertional mutagenesis, or alternative strategies of gene correction are important for all stem cell gene therapies (184).

Lentiviruses are an attractive option, since insertions occur preferentially within transcriptional units but not immediately surrounding the transcriptional start site (185). In the last years, significant advances have been made in the study and the clinical application of GT with lentiviral vectors (LV), that have been attempted with benefit and a good safety profile to the treatment not only of PIDs. Among the other diseases in which this approach has been attempted there are hemoglobinopathies, as beta (β)-thalassemia and sickle cell disease (17) and metabolic diseases, as Xlinked Adrenoleukodystrophy (18) and metachromatic leukodystrophy (19). In the PIDs field, very promising results have been obtained from the treatment of WAS with LV (186) and a recent attempt is in course by Kohn and Gaspar's group of for ADA SCID (reported at ESID meeting 2014 (81).

However, the semi-random integration of viral vectors into the genome of target cells means there is always a risk, even if small, of disrupting gene expression near the insertion sites (138).

The ultimate goal for gene therapy would be to insert the therapeutic cassette in a known, safe location within the genome that would allow expression of the therapeutic gene without perturbing the expression of other genes in the vicinity (138).

New technologies to target gene insertion should enhance safety further, maintaining appropriate regulatory control of gene expression and not risking genotoxicity through ectopic vector insertion. Technologies that allow highly specific

double-stranded DNA cleavage are beginning to be used for targeted gene insertion. This can be achieved by homologous recombination of corrected gene sequences by cellular DNA repair pathways following targeted DNA breakage. Zinc finger nucleases (ZFNs), meganucleases (MN), transcription activator-like effector nucleases (TALENS) and, more recently, clustered, regularly interspaced, short palindromic repeat (CRISPR) nucleases are all being developed to create highly specific gene targeting (184).

The efficiency of gene editing using these techniques has improved notably in cell lines and certain primary cell lineages, although remains limited in primary HSC. Nonetheless, for disorders such as SCID-X1, where corrected cells acquire a notable survival advantage, gene repair by homologous recombination holds promise as the efficiency of nuclease reagents improves (138, 184).

As data from the current clinical trials become available, it is likely that gene therapy will move from an experimental approach to become part of the mainstream therapeutic cellular armament, although long term follow-up of treated patients will be required to monitor potential long-term adverse events. The adoption of vector manufacture by mainstream pharmaceutical companies is likely to accelerate the adoption of gene therapy as a standard tool of cellular therapies.

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Purine metabolism, immune reconstitution, and abdominal adipose tumor after gene therapy for adenosine deaminase deficiency

To the Editor:

Adenosine deaminase (ADA) is an important enzyme for purine metabolite degradation or salvage into the nucleotide pool. Inherited defects in the function of ADA cause accumulation of adenosine, deoxyadenosine, and total deoxyadenosine nucleotides, resulting in severe T- and B-cell immune deficiency. ADA-deficient patients often present in infancy with increased susceptibility to infections and autoimmunity.[1] Allogeneic bone marrow (BM) transplantation with HLA-matched sibling donors can correct the immune deficiency. Since 2000, gene therapy with the patient's BM cells transduced *ex vivo* with retroviruses carrying a correct ADA gene transcript has been performed in more than 30 patients.[2] After gene therapy, most ADA-deficient patients had a significant reduction in blood levels of toxic purine metabolites, with progressive immune reconstitution.[2] Malignant cell transformation has not been reported with gene therapy for ADA deficiency in contrast to other immune deficiency diseases, such as IL-2 receptor γ deficiency and chronic granulomatous disease. Herein we have carefully evaluated purine metabolism and immune reconstitution in a patient with ADA deficiency who had an abdominal tumor 3 years after gene therapy.

The patient presented at 1 month of age with increasing respiratory difficulties, lymphopenia, and reduced T- and B-cell numbers and function (see Table E1 in this article's Online Repository at www.jacionline.org). ADA activity in his red blood cells (RBCs) was less than 1% of normal control values. Sequencing of the patient's ADA gene revealed compound heterozygosis, including a G216R substitution on one allele and 30698_30702delGAAGA on the other, mutations that were previously described in other ADA-deficient patients. The patient had no HLA-matched related donor and was subsequently enrolled in an experimental ADA gene therapy protocol. At 16 months of age, in May 2007, the patient received nonmyeloablative conditioning with 2 mg/kg busulfan (Busilvex; Pierre Fabre, Castres, France) for 2 days, followed by an infusion of BM-derived $CD34^+$ cells transduced with the ADA-containing vector in accordance with a clinical trial protocol, as previously described.[2]

Three years after gene therapy, the patient had not had any significant infections, his developmental milestones were appropriate for his age, and his height and weight were normal. The ADA activity in his peripheral blood lymphocytes increased to 402 nmol/h/mg protein (normal value, 2100 ± 320 nmol/h/mg protein). ADA activity in the patients' peripheral RBCs was about 5% of the normal value (4 nmol/min/mL; normal control value, 55-194 nmol/min/mL). Deoxyadenosine in the urine, which was 26.3 mmol/mol creatinine before treatment, had decreased to 4.4 mmol/mol creatinine, although it was still increased (normal value, <0.1 mmol/mol creatinine). Immune evaluations revealed low numbers of circulating $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, with *in vitro* responses to mitogens and antigens comparable with normal control values (see Table E1). Analysis of the repertoire of T-cell receptors among $CD3^+$ cells was normal; however, a more detailed evaluation demonstrated reduced representation of Vβ4 and Vβ5.2 families among the $CD4^+$ subpopulation, as well as reduced Vβ5.3, Vβ7.1, and Vβ9 representation but expanded Vβ5.2 and Vβ13.1 representation among the $CD8^+$ cells (see Fig E1 in this article's Online Repository at www.jacionline.org). This might be explained, at least in part, by reduced thymic output, as evident by the patient's low T-cell receptor excision circle levels. The patient also had a reduced number of $CD19^+$ B cells and required intravenous immunoglobulin replacement. Among peripheral lymphocytes, ADA gene–corrected cells were detected in 61.1%, 73.0%, 44.3%, and 65.6% of $CD4^+$, $CD8^+$, $CD19^+$, and natural killer cells, respectively, indicating a mixed population of transduced, as well as native lymphocytes.

Routine abdominal ultrasound 3 years after gene therapy revealed a previously undetected large abdominal tumor, with the patient asymptomatic. Magnetic resonance imaging showed a well-defined right-sided 4.1 × 7.0 × 7.3–cm tumor within the mesentery and below the liver (Fig 1). The mesenteric tumor and an attached segment of the small bowel were removed. The tumor was well circumscribed, thinly encapsulated, and confined to the mesentery (Fig 1). The cut surface was lobulated, soft, and yellow, with a fatty appearance. Microscopic examination revealed a tumor composed of mature adipocytes with slight variation in cell size and no cytologic atypia or immature adipocytes, which is consistent with a benign lipoma (Fig 1). The tumor was nearly devoid of lymphocytes but did contain small capillaries. Cytogenetic analysis by means of G-banding showed a balanced inverted insertion of chromosome region 8q11.23-8q24 from one chromosome 8 into the long arm of one chromosome 19 at 19q13.13 (Fig 1). The rearrangement was seen in all 30 adipocytes examined. The translocation was not present in the patient's BM, lymphoid cells, or skin cells obtained before and after gene therapy. Quantitative PCR analysis for the presence of the viral construct used for gene therapy was detected in 3.4% of the tumor cell DNA, likely representing ADA gene–transduced peripheral blood cells in the tumor blood vessels.

Adipocyte tumors include a spectrum of abnormally proliferating mesenchymal cells, ranging from benign lipomas and lipoblastomas to malignant liposarcomas. Lipoblastomas are often present in childhood as a rapidly growing tumor in the extremities or trunk. Abdominal lipoblastomas comprise less than 10% of all cases and are usually localized to the retroperitoneum, whereas rare cases have been reported to arise in the mesentery. Lipoblastomas are characterized cytogenetically by rearrangement of chromosome 8q11-13, which has been identified in the vast majority of cases, similar to the patient described here.[3,4] The observation that the tumor was of nonhematopoietic origin and that DNA from tumor cells did not contain viral gene sequences with the exception of a minor contaminant argued against a role for gene therapy–related insertional mutagenesis in this tumor formation. Another potential predisposing factor for the tumor was the use of busulfan, which has been associated with secondary malignancies; however, a link between this drug and lipoid tumors has never been reported.

Large lipoid tumors have not been described previously in ADA-deficient patients; however, these patients tend to have hematologic malignancies.[5] Moreover, ADA-deficient patients also were reported to acquire genetic abnormalities, including

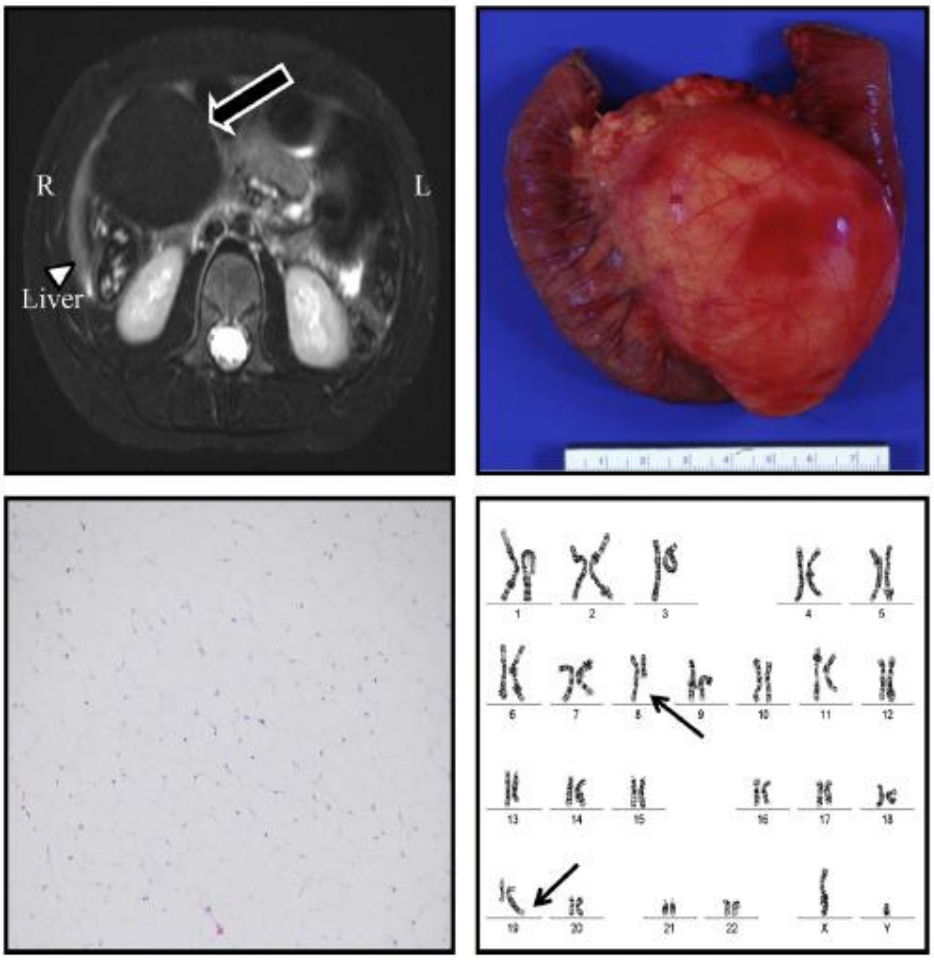

FIG 1. Analysis of the abdominal tumor. Magnetic resonance imaging *(upper left panel)* demonstrated a large right-sided abdominal tumor *(arrow)* near the liver *(arrowhead)*. The tumor had a diffuse, homogeneous high signal on T1 and T2 sequences isointense to the subcutaneous fat, with no evidence of cystic changes or calcification. Gross inspection of the tumor revealed a well-circumscribed, thinly encapsulated tumor confined to the mesentery *(upper right panel)*. Hematoxylin and eosin staining of the resected tumor demonstrated mature adipocytes with no cytologic atypia or mitoses *(lower left panel)*. G-banding analysis of the resected tumor showed balanced inverted insertion of 8q11.23-q24 into chromosome 19 at 19q13.13, which is typical of lipoblastoma *(lower right panel)*.

trisomy 8, as well as 17q22 to 22q13 translocations, which have been linked to extensive dermatofibrosarcoma protuberans.[6] The increased frequency of tumors in patients with ADA deficiency might be related to nucleotide imbalances, which impair DNA recombination in ADA-deficient lymphocytes[7] and might contribute to the increased irradiation sensitivity of ADA-deficient fibroblasts.[8] Indeed, our patient had increased concentrations of deoxyadenosine in the urine and total deoxyadenosine nucleotides in RBCs, indicating continued abnormal purine homeostasis similar to that found in ADA-deficient patients who had dermatofibrosarcoma protuberans and lymphoma after BM transplantation or PEG-ADA replacement therapy.[5,6]

Alternatively, incomplete immune reconstitution, as suggested by the reduced thymic output, skewed T-cell repertoire, low numbers of circulating B cells, and requirement of intravenous immunoglobulin, might have led to inappropriate immune surveillance and tumor formation. Similar immune dysregulation has been linked to onset of autoimmunity and increased risk of cancer development.[9] In addition, because similar metabolic and immune abnormalities, including skewed T-cell repertoire and reduced T-cell receptor excision circle levels, are frequently observed in ADA-deficient patients after BM transplantation and enzyme replacement therapy,[5] they too need to be followed closely for development of tumors.

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In conclusion, gene therapy has thus far efficiently protected our patient from significant infections and supported normal growth and development. However, the degree of correction of purine metabolism and immune reconstitution might either predispose to or inadequately protect against noninfectious morbidities.

Eyal Grunebaum, MD[a,b]
Catherine T.-S. Chung, MD[c]
Harjit Dadi, PhD[a]
Peter Kim, MD, PhD[b,d]
Immacolata Brigida, BSc[e]
Francesca Ferrua, MD[f]
Maria P. Cicalese, MD[f]
Alessandro Aiuti, MD[e,f]
Chaim M. Roifman, MD[a]

From [a]the Division of Allergy and Clinical Immunology, [b]Developmental and Stem Cell Biology, [c]the Division of Pathology, Department of Pediatric Laboratory Medicine, and [d]the Department of Surgery, The Hospital for Sick Children, Toronto, Ontario, Canada; [e]the San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan, Italy; and [f]the University of Rome Tor Vergata, Rome, Italy. E-mail: Eyal.grunebaum@sickkids.ca.

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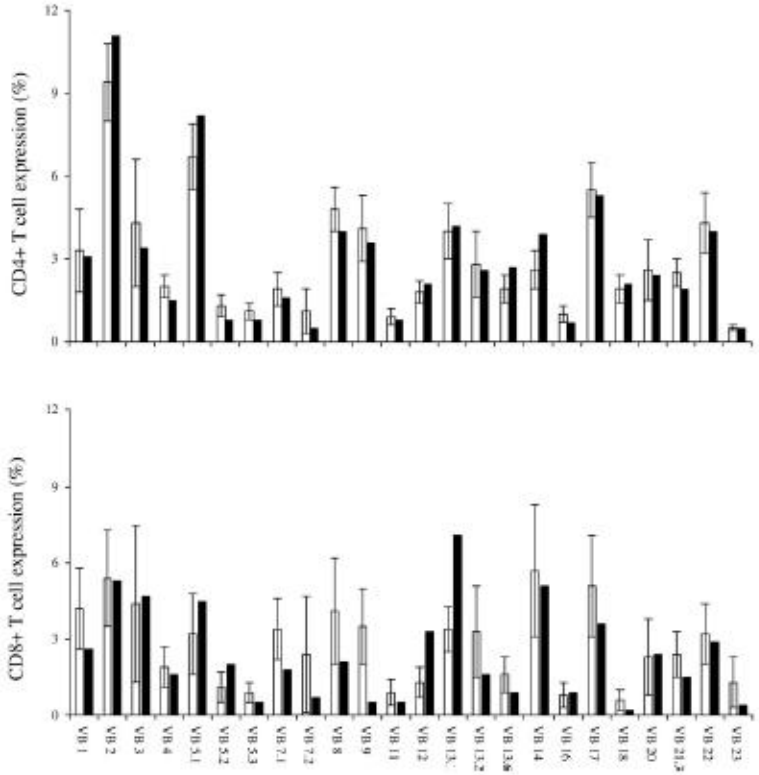

FIG E1. T-cell receptor diversity. Relative expression of the various Vβ families among $CD4^+$ *(upper panel)* and $CD8^+$ *(lower panel)* T cells in the patient 3 years after gene therapy *(solid bars)* or normal age-matched control subjects (*open bars* ± SD), as determined by means of fluorescence-activated cell sorting analysis with the IOTest Beta Mark Kit (Beckman Coulter, Mississauga, Ontario, Canada).

TABLE E1. The patient's immune evaluations before and after gene therapy*

	At diagnosis	Age-matched healthy control subjects	Three y after gene therapy	Age-matched healthy control subjects
White blood cells ($\times 10^9$/L)	3.5	5-15	5.9	5-12
Neutrophils ($\times 10^9$/L)	2.9	1.5-8.5	3.6	1.5-8.5
Lymphocytes ($\times 10^9$/L)	0.18	4-10.5	1.2	1.5-8.0
$CD3^+$ cells ($\times 10^9$/L [%])	0.03 (20)	2.5-5.5† (53-84)	0.86 (87)	1.4-3.7† (56-75)
$CD4^+$ cells ($\times 10^9$/L [% lymphocytes])	0.02 (13)	1.6-4.0† (35-64)	0.56 (46)	0.7-2.2† (28-47)
$CD8^+$ cells ($\times 10^9$/L [% lymphocytes])	0.01 (3)	0.56-1.7† (12-28)	0.31 (25)	0.49-1.3† (16-30)
$CD19^+$ cells ($\times 10^9$/L [% lymphocytes])	0.01 (7)	0.3-2.0† (6-32)	0.11 (9)	0.39-1.4† (14-33)
$CD16/CD56^+$ cells ($\times 10^9$/L [% lymphocytes])	0.03 (20)	0.17-1.1† (4-18)	0.05 (5)	0.13-0.7† (4-17)
SI to anti-CD3	NA	NA	500	413
SI to PHA	1	366	603	667
SI to herpes simplex virus	NA	NA	18	30
T-cell receptor excision circles/0.5 μg	NA	NA	47	>350

NA, Not available; *SI*, stimulation index.
*Immunologic evaluations were performed at The Hospital for Sick Children, Toronto, Ontario, Canada, and were beyond the 3-year follow-up investigations required by the clinical trial.
†Tenth and 90th percentiles in healthy age-matched control subjects.[E1]

Immune deficiencies, infection, and systemic immune disorders

B-cell development and functions and therapeutic options in adenosine deaminase–deficient patients

Immacolata Brigida, PhD,[a] Aisha V. Sauer, PhD,[a] Francesca Ferrua, MD,[c] Stefania Giannelli, PhD,[a] Samantha Scaramuzza, PhD,[a] Valentina Pistoia, MSc,[a] Maria Carmina Castiello, PhD,[a,d] Barbara H. Barendregt, BSc,[e] Maria Pia Cicalese, MD,[c] Miriam Casiraghi, RCN,[c] Chiara Brombin, PhD,[f] Jennifer Puck, MD,[g] Klaus Müller, MD, PhD,[h] Lucia Dora Notarangelo, MD,[i] Davide Montin, MD,[j] Joris M. van Montfrans, MD, PhD,[k] Maria Grazia Roncarolo, MD,[a,d] Elisabetta Traggiai, PhD,[l] Jacques J. M. van Dongen, MD, PhD,[e] Mirjam van der Burg, PhD,[e] and Alessandro Aiuti, MD, PhD[a,b] *Milan, Rome, Brescia, and Turin, Italy, Rotterdam and Utrecht, The Netherlands, San Francisco, Calif, Copenhagen, Denmark, and Basel, Switzerland*

Background: Adenosine deaminase (ADA) deficiency causes severe cellular and humoral immune defects and dysregulation because of metabolic toxicity. Alterations in B-cell development and function have been poorly studied. Enzyme replacement therapy (ERT) and hematopoietic stem cell (HSC) gene therapy (GT) are therapeutic options for patients lacking a suitable bone marrow (BM) transplant donor.

Objective: We sought to study alterations in B-cell development in ADA-deficient patients and investigate the ability of ERT and HSC-GT to restore normal B-cell differentiation and function.

Methods: Flow cytometry was used to characterize B-cell development in BM and the periphery. The percentage of gene-corrected B cells was measured by using quantitative PCR. B cells were assessed for their capacity to proliferate and release IgM after stimulation.

Results: Despite the severe peripheral B-cell lymphopenia, patients with ADA-deficient severe combined immunodeficiency showed a partial block in central BM development. Treatment with ERT or HSC-GT reverted most BM alterations, but ERT led to immature B-cell expansion. In the periphery transitional B cells accumulated under ERT, and the defect in maturation persisted long-term. HSC-GT led to a progressive improvement in B-cell numbers and development, along with increased levels of gene correction. The strongest selective advantage for ADA-transduced cells occurred at the transition from immature to naive cells. B-cell proliferative responses and differentiation to immunoglobulin secreting IgM after B-cell receptor and Toll-like receptor triggering were severely impaired after ERT and improved significantly after HSC-GT.

Conclusions: ADA-deficient patients show specific defects in B-cell development and functions that are differently corrected after ERT and HSC-GT. (J Allergy Clin Immunol 2014;133:799-806.)

Key words: *Gene therapy, adenosine deaminase–deficient severe combined immunodeficiency, B-cell development, antibodies*

Mutations in the adenosine deaminase (ADA) gene are associated with accumulation of its substrates adenosine and deoxyadenosine, leading to lymphopenia (T, B, and natural killer) and a wide spectrum of immune and nonimmune alterations.[1,2] Bone marrow transplantation (BMT) from an HLA-identical sibling donor is the treatment of choice for ADA-deficient severe combined immunodeficiency (SCID), but transplants from matched unrelated donors are associated with increased morbidity and mortality.[3] Enzyme replacement therapy (ERT) with PEG-ADA decreases toxic ADA substrate concentrations and improves the immunologic phenotype. Nonetheless, a variable extent of immune recovery has been reported, and patients undergoing long-term treatment often show a decrease in lymphocyte counts, loss of regulatory T (Treg) cell function, and development of antibodies against bovine ADA.[2,4-7] Gene therapy (GT) with hematopoietic stem cells (HSCs) engineered with gamma retroviral vectors has been shown to be a successful alternative strategy for patients who do not have access to HLA-identical BMT and for whom ERT was insufficient to maintain adequate immune reconstitution.[8] Since 2000, more than 40 patients worldwide were enrolled in HSC-GT clinical trials with reduced-intensity conditioning, resulting in long-term multilineage engraftment, sustained systemic detoxification, and improved immune functions.[8-11]

The lack of ADA induces severe peripheral B-cell lymphopenia, but no information is available about bone marrow (BM) B-cell development in patients with untreated ADA-SCID. Most

From [a]San Raffaele Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute, Milan; [b]the Department of Systems Medicine, Tor Vergata University, Rome; [c]Pediatric Immunohematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan; [d]Vita-Salute San Raffaele University, Milan; [e]the Department of Immunology, Erasmus MC, University Medical Center, Rotterdam; [f]CUSSB, Vita-Salute San Raffaele University, Milan; [g]the Division of Allergy, Immunology and Bone Marrow Transplantation, Department of Pediatrics, University of California San Francisco; [h]Pediatric Clinic, Juliane Marie Center, Copenhagen; [i]the Pediatric Onco-Hematology and BMT Unit, Children's Hospital, Spedali Civili, Brescia; [j]the Department of Pediatrics, University of Turin; [k]the Department of Pediatric Immunology and Infectious Diseases, University Medical Center Utrecht; and [l]the Novartis Institute for Research in Biomedicine, Basel.

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Corresponding author: Alessandro Aiuti, MD, PhD, TIGET, Via Olgettina 58, 20132 Milano, Italy. E-mail: aiuti.alessandro@hsr.it.

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Abbreviations used
ADA: Adenosine deaminase
ANA: Anti-nuclear antibody
APC: Allophycocyanin
BAFF: B cell–activating factor
BAFF-R: BAFF receptor
BCR: B-cell receptor
BM: Bone marrow
BMT: Bone marrow transplant
CD40L: CD40 ligand
CFSE: Carboxyfluorescein succinimidyl ester
ERT: Enzyme replacement therapy
FACS: Fluorescence-activated cell sorting
FITC: Fluorescein isothiocyanate
GT: Gene therapy
HSC: Hematopoietic stem cell
IVIg: Intravenous immunoglobulin
LME: Linear mixed effect
PB: Peripheral blood
PE: Phycoerythrin
qPCR: Quantitative PCR
SCID: severe combined immunodeficiency
TLR: Toll-like receptor
Treg: Regulatory T

patients treated with ERT show an initial B-cell recovery that is not sustained long-term, with reduction in newly produced B-cell counts and oligoclonal B-cell repertoire.[7,12,13] Additionally, patients with milder forms of ADA deficiency after treatment with PEG-ADA or HSC-GT have shown autoimmune manifestations, which might be related to central or peripheral B-cell tolerance defects.[1,2,8,14,15]

B-cell development is regulated by the elimination of self-reactive B-cell clones in the BM and during transition from new emigrant to mature naive B-cell stages in the periphery.[16,17] B cells from patients with ADA-SCID show a high frequency of autoreactive and anti-nuclear antibody (ANA)–expressing clones, loss of central and peripheral B-cell tolerance, and perturbation of checkpoint control during development.[18] Moreover, patients with different primary immunodeficiencies who have autoimmunity show impaired elimination of autoreactive B cells at the transitional B-cell stage[19,20] and an altered distribution of autoimmune-prone B-cell subsets, including $CD21^{low}CD38^{low}$ B cells.[21]

These observations led us to investigate alterations in B-cell development in ADA-deficient patients and to compare B-cell development and function in patients with ADA-SCID receiving either ERT, HSC-GT, or BM transplantation.

METHODS

The methodology used in this study is described in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Altered BM B-cell development in patients with ADA-SCID and correction after treatment

To gain information on early B-cell development, we studied the composition of B-cell precursors in the BM of 7 patients with untreated ADA-SCID, 6 ERT-treated patients, and 8 patients undergoing HSC-GT (see Table E1 in this article's Online Repository at www.jacionline.org). We used 13 combinations of 4-color staining to identify pro-B, pre-BI, pre-BII, and immature B cells (Fig 1, *A*).[22,23]

Despite the severe peripheral lymphopenia in patients with untreated ADA-SCID, B cells were present during all stages of BM maturation (Fig 1). Pro-B cells were similarly increased in untreated, ERT-treated, and HSC-GT–treated patients (Fig 1, *B*). Strikingly, a 2-fold increase in pre-BI–cell counts was observed in patients with untreated ADA-SCID compared with control subjects (48% ± 6% vs 18.5% ± 7%, respectively [mean ± SD]), with a corresponding decrease in pre-BII and immature B-cell counts. This suggests a progressive loss of mature precursor cells rather than a block in differentiation. Treatment with ERT reduced the percentage of pre-BI cells, with a corresponding recovery in pre-BII–cell counts. Immature B-cell counts were also increased, suggesting an altered progression of B-cell development through maturation. Importantly, the proportion of pre-BI, pre-BII, and immature B-cell subsets was normalized after HSC-GT (Fig 1, *C-E*, and see Fig E1 in this article's Online Repository at www.jacionline.org).

Selective advantage for gene-corrected B cells occurs at late stages of maturation

In agreement with our previous observations,[8] on average, the proportion of vector-transduced B cells in patients undergoing HSC-GT was 8.9% (range, 0.6% to 32.4%) in BM $CD19^+$ cells and 48.3% (range, 8.4% to 76.4%) in peripheral blood (PB). Therefore we assessed whether the observed selective advantage for ADA-transduced B cells occurred during BM development or at later stages of maturation. Pro-B, pre-BI, pre-BII, and immature B cells from 6 patients (collected 2.6-8.3 years after HSC-GT) were sorted by means of fluorescence-activated cell sorting (FACS) and analyzed by using quantitative PCR (qPCR) for their percentage of gene correction (Fig 2, *A*). No significant increase in the fraction of gene-corrected progenitor B cells was observed during the early stages of development (Fig 2, *A*, left panel), whereas the percentage of transduced naive B cells increased, on average, 4.4-fold (Fig 2, *A*, right panel; median, 24.5%) with respect to immature B cells in the BM (5.6%). In addition to the selective advantage occurring at the transition from immature to naive B cells, we observed a further increase at later stages of maturation because approximately 56% of total $CD19^+$ B cells were gene corrected in these patients (Fig 2, *B*).

Transitional B cells accumulate during ERT treatment

Patients who underwent ERT were divided into short-term (n = 8; 0.4-2.5 years) and long-term (n = 6; 9.1-22.8 years) categories based on their age and duration of treatment. B-cell counts were less than the normal range[24] in both the short- and long-term groups (see Table E2 in this article's Online Repository at www.jacionline.org). Nevertheless, all patients undergoing long-term ERT discontinued intravenous immunoglobulin (IVIg) and responded to vaccine antigens. After HSC-GT, B-cell counts were normal in 3 of 14 patients, and 71% of patients discontinued IVIg with good vaccination response. Three patients undergoing ERT and 2 patients undergoing HSC-GT showed signs of autoimmunity (see Table E2). ANA results were positive in 2 asymptomatic patients undergoing HSC-GT, whereas 2

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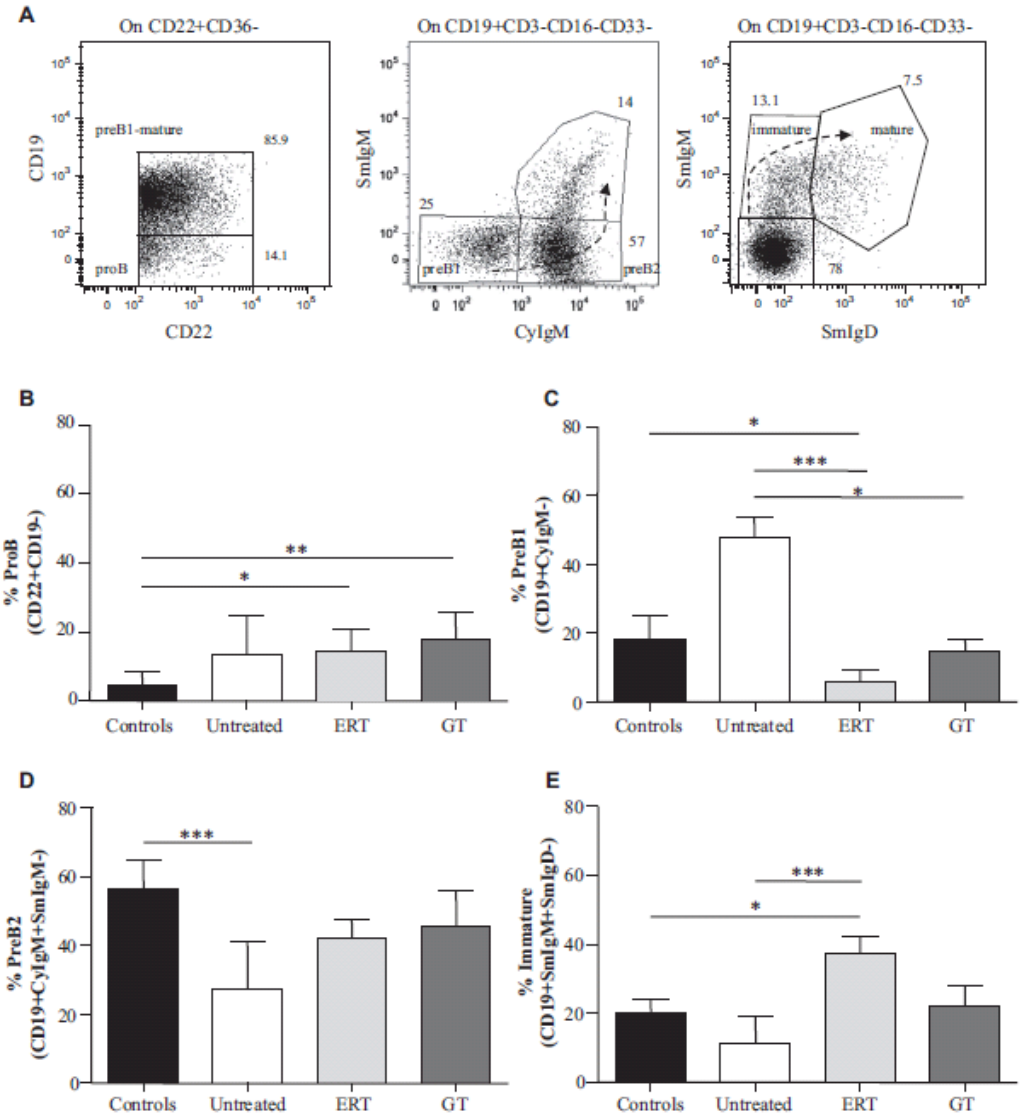

FIG 1. BM B-cell development in patients with ADA-SCID. **A**, Representative FACS staining and gating strategy for BM B-cell development. *Dotted arrows* indicate the stage of development of pro-B ($CD22^+CD19^-$), pre-BI ($CD19^+CyIgM^-SmIgM^-$), pre-BII ($CD19^+CyIgM^+SmIgM^-$), immature ($CD19^+SmIgM^+SmIgD^-$), and mature ($CD19^+SmIgM^+SmIgD^+$) B cells. **B-E**, Percentage of pro-B (Fig 1, *B*), pre-BI (Fig 1, *C*), pre-BII (Fig 1, *D*), and immature (Fig 1, *E*) B cells in 10 age-matched healthy donors, 7 patients with ADA-SCID, 6 patients undergoing ERT, and 8 patients undergoing HSC-GT corrected for blood contamination. $*P < .05$, $**P < .005$, and $***P < .001$, Kruskal-Wallis test with Dunn correction.

patients undergoing ERT showed ANA or Coombs positivity (data not shown).

To understand whether different treatments influence B-cell development, we compared the proportion and absolute numbers of PB B-cell subsets of patients undergoing HSC-GT with those of patients undergoing ERT and appropriate age-matched control subjects. The GT cohort was subdivided in 2 categories according to the years of follow-up (1-4 and 4.6-8.9). As shown in Fig 3, the percentage of $CD24^{hi}CD38^{hi}$ transitional B cells was 5.8-fold higher than in control subjects in both the short-term and long-term ERT groups ($P = .0001$; Fig 3, *B*), whereas absolute counts were increased only after short-term treatment ($P = .001$;

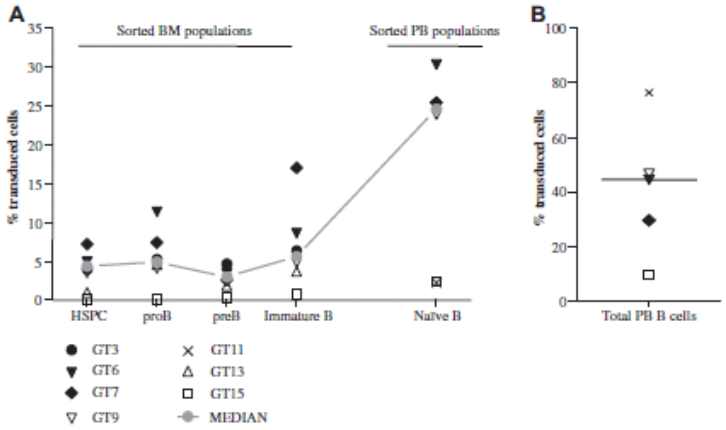

FIG 2. Selective advantage of gene-corrected B cells occurs at late stages of maturation. **A,** Percentage of transduced BM and PB B-cell subpopulations determined by means of qPCR (BM, n = 6; PB, n = 5). *Gray line,* Median values. **B,** Percentage of gene-corrected B cells in PB. *Bar* indicates median value.

Fig 3, *C*). The percentage of transitional B cells was increased only in the short-term group of patients undergoing HSC-GT but not the absolute numbers ($P = .007$; Fig 3, *B*). Patients undergoing HSC-GT normalized both transitional B-cell numbers and percentages, as assessed by using a linear mixed effect (LME) model (Fig 3, *D*), whereas this alteration persisted after long-term ERT ($P = .2232$; Fig 3, *D*). The percentage of transitional B cells in patients undergoing HSC-GT inversely correlated with transduced B cells ($P = .0401$; Fig 3, *E*), indicating that the relatively increased transitional B-cell percentages were mainly nontransduced.

We then measured the proportion of naive ($CD19^+CD27^-$), $CD27^+$ memory, and switched memory ($CD27^+IgG^+IgA^+$) B cells (see Fig E2 in this article's Online Repository at www.jacionline.org). Short-term ERT resulted in normal naive B-cell counts, but the long-term generation of naive B cells was not sustained. Decreased memory and switched memory B-cell counts were persistently found in patients receiving short- and long-term ERT (see Fig E2, *D* and *F*). In contrast, all B-cell subpopulations were reduced at early time points after HSC-GT compared with those seen in control subjects but normal in patients followed long-term ($P = .0003$, $P = .002$, and $P = .003$, respectively; see Fig E2, *B*, *D*, and *F*).

We next analyzed the $CD21^{low}CD38^{low}$ B-cell subset, which was previously shown to be expanded in patients with autoimmunity or immunodeficiency.[21,25] This population was overrepresented in patients undergoing long-term ERT (8%, $P < .005$) and in patients undergoing HSC-GT ($P = .04$) when compared with control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org). However, no association with autoimmune manifestations was observed in both groups of patients.

Effect of B cell–activating factor on transitional B-cell maturation

B-cell survival, peripheral selection, and maturation largely depend on the activity of B cell–activating factor (BAFF).[26] BAFF plasma levels were evaluated in patients undergoing ERT and patients undergoing HSC-GT during follow-up. Shortly after PEG-ADA, BAFF levels were increased ($P < .05$; see Fig E4, *A*, in this article's Online Repository at www.jacionline.org), whereas long-term treatment resulted in levels comparable with those seen in control subjects (median, 0.7 ng/mL; range, 0.4-2 ng/mL). Also, patients undergoing HSC-GT showed increased levels of BAFF (7-fold with respect to control subjects), but its concentration decreased over time ($P < .05$). We then evaluated the level of BAFF receptor (BAFF-R) expression, which has a pivotal role in regulating the size of the mature B-cell pool (see Fig E4, *B*). We observed a 2.8- and a 3.8-fold reduction in BAFF-R mean fluorescence intensity in patients undergoing short-term HSC-GT and ERT (see Fig E4, *C*), which is in accordance with higher BAFF levels and a more immature B-cell phenotype in both groups. BAFF-R instead normalized in the long-term HSC-GT group, which is in accordance with a normalization of BAFF in their plasma.

Reduced *in vitro* proliferation of B cells from ERT-treated patients is corrected after GT

To study the ability of B cells from patients with ADA-SCID to proliferate and differentiate *in vitro*, we purified $CD20^+$ B cells and stimulated them through the B-cell receptor (BCR) or Toll-like receptor (TLR). Fig E5 in this article's Online Repository at www.jacionline.org shows carboxyfluorescein succinimidyl ester (CFSE) dilutions in a representative control subject, patients undergoing HSC-GT, and patients undergoing ERT after stimulation with the TLR9 agonist CpG or in combination with immunoglobulin or CD40 ligand (CD40L). TLR stimulation did not induce adequate proliferation in B cells from patients undergoing ERT (Fig 4, *A*), indicating that TLR receptors are unable to signal properly in the absence of functional ADA. The additional BCR stimulation by anti-immunoglobulin antibody was unable to restore appropriate B-cell proliferation in patients undergoing ERT ($P = .003$). In contrast, B cells from patients undergoing HSC-GT responded normally after CpG stimulation, and costimulation of the BCR further increased B-cell proliferation. The addition of CD40L to mimic T-cell/B-cell interaction induced adequate B-cell proliferation in patients undergoing HSC-GT but not patients undergoing ERT. Because the level of gene correction varies between patients, we analyzed patients with different percentages

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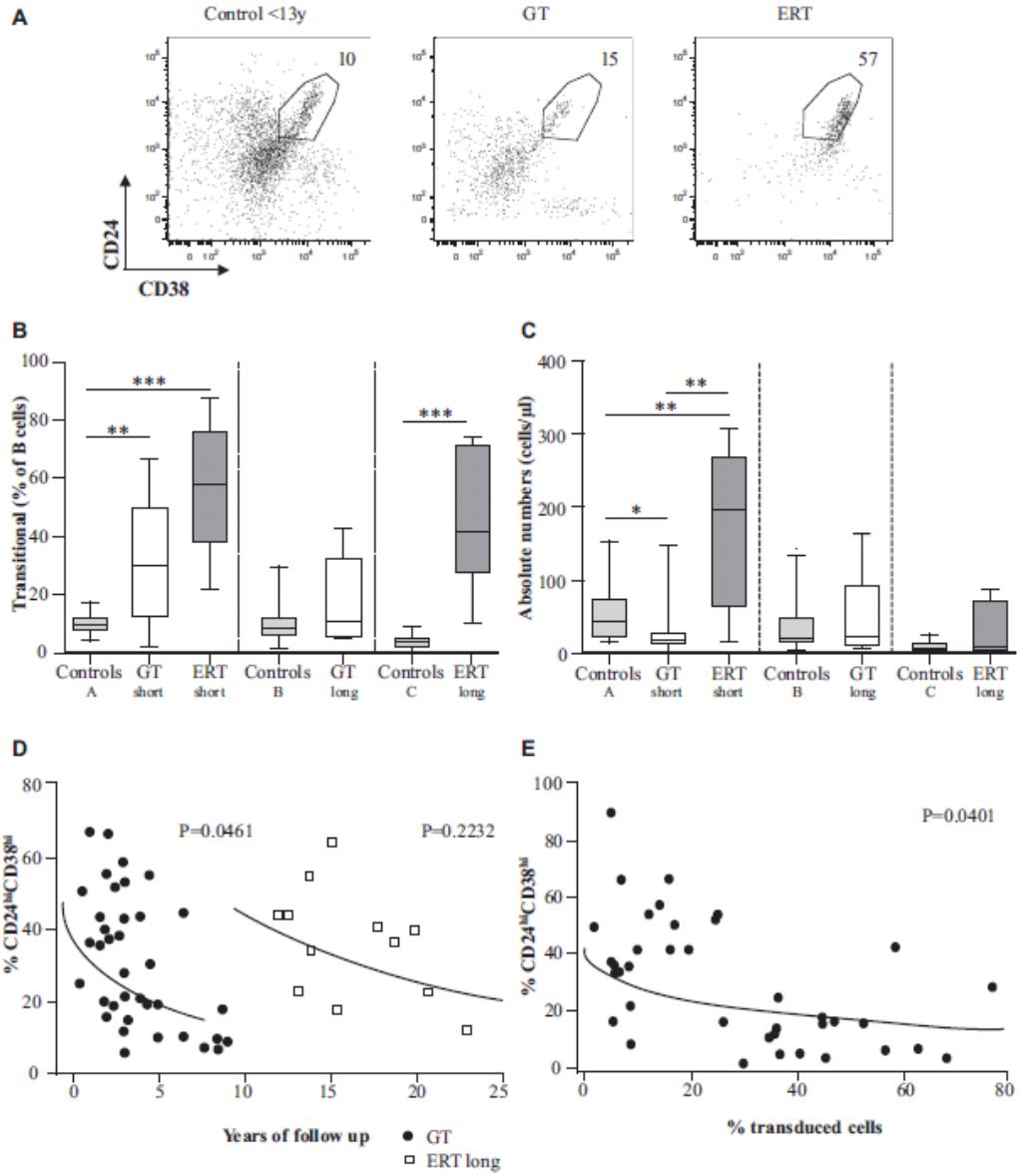

FIG 3. Transitional B cells accumulate in patients undergoing ERT and decrease after HSC-GT. **A,** Representative dot plots for age-matched control subjects, patients undergoing HSC-GT, and patients undergoing ERT for $CD24^{hi}CD38^{hi}$ transitional B cells. **B,** Percentage of transitional B cells in patients undergoing short-term HSC-GT (n = 8), patients undergoing short-term ERT (n = 8), patients undergoing long-term HSC-GT (n = 6), and patients undergoing long-term ERT (n = 6). Age-matched control subjects are shown: Controls A (n = 24, 0.5-4 years), Controls B (n = 36, 4.1-13 years), and Controls C (n = 30, 13-25 years). Median values with 5th and 95th percentiles. *$P < .05$ and **$P < .005$, Mann-Whitney test. **C,** Absolute numbers of transitional B cells in the same groups as in Fig 3, *B*, are shown. Data are presented as median values with 5th and 95th percentiles. *$P < .05$, **$P < .005$, and ***$P < .001$, Mann-Whitney test. **D,** Longitudinal analysis for the percentage of transitional B cells and years of F.U. for 14 patients undergoing HSC-GT *(black dots)* and 5 ERT-treated patients *(open squares)*. Analysis of longitudinal data was done with LME models and time effect significance. **E,** Longitudinal analysis between the percentage of transitional B cells and the percentage of vector-transduced B cells in the 14 HSC-GT–treated patients studied using LME models, with significance.

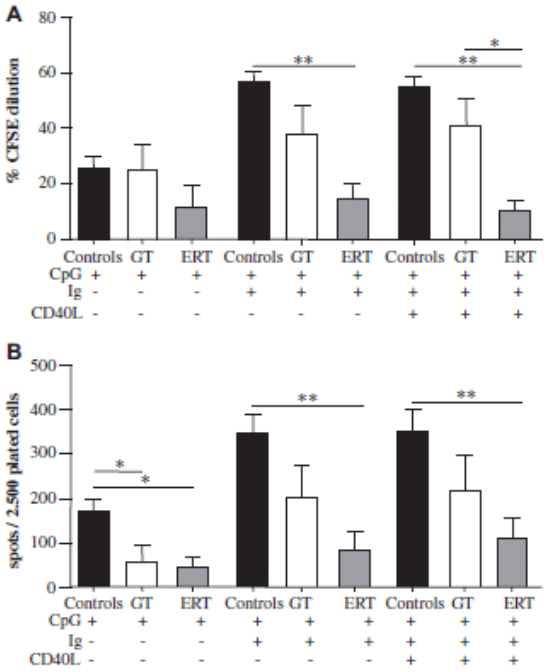

FIG 4. Impaired B-cell proliferation after BCR/TLR triggering in patients undergoing ERT is corrected after HSC-GT. **A,** Percentage of CFSE-diluting B cells in patients undergoing HSC-GT (n = 4 for CpG stimulation and n = 6 when either immunoglobulin or CD40L were added) and 4 ERT-treated patients compared with 15 control subjects. Data are shown as means ± SEMs. *P < .05 and **P < .005, Mann-Whitney test. **B,** Number of IgM-producing B cells in patients undergoing ERT (n = 5) and patients undergoing HSC-GT (n = 5 for CpG stimulation and n = 6 when either immunoglobulin or CD40L were added) compared with 17 control subjects. Data are shown as means ± SEMs. *P < .05 and **P < .005, Mann-Whitney test.

of transduced B cells separately. Their ability to respond to a combination of BCR/TLR stimuli and T-cell mimicking (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org) is 2-fold reduced in patients with few corrected B cells (<50%) compared with patients with high correction (>50%) or a patient successfully treated with BMT achieving full donor chimerism (see Fig E6, *B*). This finding highlights the critical importance of ADA for the normalization of B-cell function. After BCR, TLR, and CD40L activation, B cells differentiate into immunoglobulin-secreting cells. Therefore we assessed the percentage of IgM immunoglobulin-secreting cells in patients undergoing ERT or patients undergoing HSC-GT by using the ELISPOT assay (Fig 4, *B*). B cells from patients undergoing ERT and patients undergoing HSC-GT stimulated with CpG showed a reduced percentage of IgM-secreting spots with respect to control subjects. After additional BCR engagement, CD40L, or both, we observed a significant improvement in IgM secretion for the HSC-GT group and, to a lesser extent, the ERT group.

DISCUSSION

The goal of the present work was to assess defects in B-cell differentiation and function in patients with ADA deficiency and to evaluate the effect of different treatments. Here we demonstrate that patients with ADA-SCID display specific alterations in early and late B-cell development, which are differently corrected by ERT or HSC-GT. Moreover, we show that intrinsic ADA expression provided by gene transfer is superior to ERT in restoring B-cell differentiation and function (Table I).

Our findings indicate that, despite the severe peripheral lymphopenia, ADA deficiency only leads to a partial block in BM differentiation. After pre-BCR rearrangement and completion of V(D)J recombination, a 2-fold decrease in late developmental stages can be observed in patients with untreated ADA-SCID (see Fig E1), suggesting that B cells become more sensitive to purine toxic metabolites and apoptosis during maturation.[27] These alterations are possibly related to defects in V(D)J recombination and DNA damage repair caused by the accumulation of ADA substrates. In fact, previous reports indicate that increased dATP levels influence the frequency of V(D)J recombination and the composition of N insertions in lymphocytes, leading to alterations in antigen receptor and aberrant lymphoid development.[28] Moreover, increased intracellular dATP in the absence of deamination retards DNA repair in human lymphocytes and results in the slow accumulation of DNA strand breaks.[29] This is consistent with the pattern observed in patients with SCID with a complete defect in V(D)J recombination (eg, recombination-activating gene 1 or 2 deficiency) or X-linked agammaglobulinemia, showing an earlier and more severe block at pro-B and pre-BI stages and a dramatic reduction in immature B-cell counts.[22,30,31] The hypothesis that human B cells require different levels of ADA during differentiation is supported by the progressive increase of vector-transduced B cells in patients undergoing HSC-GT from immature to naive B cells and even further in late stages.

We found that both HSC-GT and ERT revert the partial BM differentiation block, leading to rescue of the peripheral B-cell pool. Because gene-corrected cells represent approximately 10% of total BM B cells, the normalization of B-cell development is likely mediated by cross-correction from ADA-expressing B cells and other cell lineages. However, our data indicate that intrinsic ADA expression achieved by GT is important for survival, maturation, and function at later stages of differentiation.

Both patients undergoing ERT and patients undergoing HSC-GT in the first years after treatment showed a relative increase in counts of transitional B cells in the periphery, which normalized at later time points in the HSC-GT group. Also, patients who underwent allogeneic BMT showed an increased proportion of transitional B cells early after transplantation, with normalization within 1 year after treatment.[20,32] After HSC-GT, a delay in B-cell reconstitution with respect to BMT is expected because of the time needed for highly purified transduced HSCs to repopulate the BM, compete with untransduced B cells, and differentiate into functionally mature B cells. Importantly, increased B-cell counts, including memory and switched memory B cells, alongside the reduction in transitional B-cell counts was associated with an increased percentage of gene-corrected B cells in the PB.

The higher proportion of transitional B cells detected in patients undergoing long-term ERT might be sustained by the increased percentage of immature B cells exiting from their BM or the higher BAFF levels found in these patients. Similar to patients with B cell–mediated autoimmune diseases,[33-35] BAFF might act as a compensatory mechanism to the lymphopenic condition, thus

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TABLE I. Summary of the main B-cell features in the BM and the periphery of patients with untreated ADA-SCID or patients who received long-term treatment with ERT or underwent HSC-GT

		PB	
Group	**BM development**	**B-cell maturation**	**B-cell function**
Untreated ADA-SCID	↑ Pre- BI B cells Block of BM development	ND	ND
ERT	Normal BM development ↑ Immature B cells	↑ Transitional B cells ↓ Naive, memory, and switched memory B cells ↑ BAFF levels	↓ B-cell proliferation ↓ IgM production to TLR7/9 and immunoglobulin
HSC-GT	Normal BM development Selective advantage for gene-corrected cells (later stages)	Progressive normalization of B-cell maturation ↑ BAFF levels	Normal B-cell proliferation ↓ IgM production to TLR7/9

ND, Not done because of severe lymphopenia.

allowing the expansion of transitional or $CD21^{low}CD38^{low}$ B cells and promoting cell-mediated autoimmunity through survival of low-affinity self-reactive B cells.

In patients undergoing HSC-GT, B-cell development proceeded to mature functional B cells and a normal proportion of memory and immunoglobulin-producing B cells. Interestingly, the restored ability of plasmablasts to proliferate and produce IgM *in vitro* correlated with their level of transduction, indicating that endogenous ADA is required for full correction of the B-cell defect. In contrast, the few naive B cells isolated *ex vivo* from patients undergoing ERT did not properly respond to BCR or TLR stimulation, proliferate, and secrete immunoglobulins. These data are in agreement with our previous finding that *in vitro* inhibition of ADA enzymatic activity in normal human B cells blocks responses to TLR and BCR stimulation.[18]

Development of autoantibodies and autoimmune manifestations have been reported after long-term ERT[12,14,15] and have been associated with an incomplete immune recovery, decrease in absolute B-cell numbers, and an oligoclonal B-cell repertoire.[2,6,12,36] An in-depth analysis of B-cell tolerance and antibody repertoire in 3 ADA-deficient patients treated with ERT showed an increased frequency of both polyreactive and ANA-expressing clones, indicating defects in central and peripheral B-cell tolerance in patients with ADA deficiency.[2,18] Moreover, we previously demonstrated that an impaired Treg cell function contributes to the loss of peripheral tolerance in patients undergoing ERT, as well as in $ADA^{-/-}$ mice rescued with PEG-ADA therapy. In these animals the abolished Treg cell function leads to the development of immune dysregulation with abnormal serum immunoglobulin levels, antiplatelet antibodies, and autoantibodies directed against multiple organs.[5]

Herein we found a significant increase in the percentage of $CD21^{low}CD38^{low}$ B cells in patients undergoing ERT, a population highly represented among those with systemic lupus erythematosus[25] and common variable immunodeficiency,[37] resulting in enriched autoreactive clones refractory to BCR triggering and unable to upregulate activation markers. Similar to mouse T1 B cells,[38] $CD21^{low}CD38^{low}$ B cells escape central B-cell tolerance mechanisms and remain irresponsive in the periphery. Inflammatory responses might create a favorable environment to break tolerance and eventually activate $CD21^{low}CD38^{low}$ B cells.[21] An alternative explanation is that $CD21^{low}CD38^{low}$ B cells represent a developmental stage that precedes that of transitional B cells.[20] Also, patients treated with HSC-GT had a higher proportion of $CD21^{low}CD38^{low}$ cells, suggesting a possible predisposition to autoimmune manifestations because of the escape of autoreactive immature B cells that are not eliminated in the periphery.

In the present study we did not observe an increase in autoimmune manifestations or autoantibody production in the ERT group. Because of the low number of patients who had autoimmunity and the limited size of the patient cohort, a direct correlation between clinical signs and biological observations (Treg cell numbers, transitional B cells, and BAFF levels) could not be drawn in both the ERT and HSC-GT groups. A combination of predisposing factors might favor the onset of immune dysregulation. It is also plausible that infections might contribute to immune dysregulation, although we did not observe an increased infection rate in patients with autoimmunity.

In the GT cohort it is possible that systemic detoxification after GT and the metabolic cross-correction of uncorrected B cells by gene-corrected cells in BM allows the survival of potentially autoreactive B cells and their egress in the periphery. The coexistence of noncorrected autoreactive B cells, which produce higher levels of ANA *in vitro*,[18] and gene-corrected functional T-cell help might explain why some patients with ADA-SCID have autoimmune manifestations after HSC-GT. Finally, it cannot be excluded that tolerance defects established under ERT treatment are insufficiently controlled after GT.

In summary, our findings provide important insights into defects in B-cell maturation in ADA-deficient patients. These alterations are partially corrected after ERT because B cells remain mostly at the transitional stage and retain severe defects in proliferation and antibody secretion *in vitro*. GT with HSC provides progressive normalization of B-cell development with recovery of B-cell functions. The majority of treated patients were not taking IVIg, showing specific antibody responses and no autoimmune manifestations. Maintenance of long-term B-cell reconstitution remains to be determined, especially in patients with immunologic defects. Increased HSC-GT efficiency and engraftment of gene-corrected B cells could further improve immune reconstitution at early time points after follow-up and reduce the risk of autoimmunity.

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Clinical implications: HSC-GT is superior to ERT in restoring B-cell development and *in vitro* B-cell function in patients with ADA-SCID.

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METHODS

Patients with ADA-SCID and clinical trial

BM samples of naive patients with ADA-SCID were collected on the occasion of diagnostic procedures from the remaining cell material, according to the informed consent guidelines of Rotterdam University Hospital. BM of control subjects was obtained according to the informed consent guidelines of the Medical Ethics Committees of the Leiden University Medical Center. For patients undergoing ERT or those after HSC-GT, PB and BM were collected after obtaining parental informed consent on the occasion of diagnostic procedures or safety follow-up, according to the San Raffaele Hospital–approved research protocol for pathogenetic studies in immunodeficient patients.

Patients with ADA-SCID undergoing HSC-GT were enrolled in phase I/II clinical protocols approved by the San Raffaele Scientific Institute's Ethical Committee and Italian National Regulatory Authorities. HSC-GT treatment was performed as previously described.[E1] Data from patients undergoing HSC-GT were collected from 2008 to 2011. Since April 2012, GlaxoSmith-Kline has become a sponsor of ADA-SCID long-term follow-up trial no. 115611 (HSC-GT) conducted at TIGET. A previously described patient[E1] not studied in the present work showed autoimmune manifestations during PEG-ADA, which persisted after GT.

Patients undergoing short-term ERT received 19 to 80 U/kg/wk ADAGEN (Pegademase bovine; ENZON Pharmaceuticals, Piscataway, NJ) before HSC-GT, where patients undergoing long-term ERT received 10 to 40 U/kg/wk. A subgroup of patients undergoing long-term ERT had previously received (8.5-11.3 years earlier) infusion of transduced PB T cells,[E2] without evidence of long-term marking in other lineages. The patient with ADA-SCID treated with BMT was previously described.[E3]

PB from pediatric control subjects was obtained on the occasion of other blood testing after informed consent in the context of a research protocol established at San Raffaele Scientific Institute. Informed consent was approved by the Institutional Ethical Committee of San Raffaele, and forms were signed by all subjects' parents.

Isolation of blood cells, cell purification, and qPCR

PBMCs and BM mononuclear cells were purified by using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient separation. The protocol for the purification of different cell subsets and qPCR for gene-corrected cells was performed as previously described.[E4] The purity of $CD19^+$ B cells was greater than 90%.

FACS staining and B-cell sorting

BM progenitor B cells were analyzed on thawed samples from patients with untreated ADA-SCID and patients undergoing ERT or after HSC-GT. Data were compared with reference values obtained from 10 age-matched control subjects (Table E1).

Frozen mononuclear cells from patients with ADA SCID were thawed in RPMI and 10% FBS and directly incubated with 100 μL of the following mix of antibodies: CD34 fluorescein isothiocyanate (FITC), CD22 allophycocyanin (APC), CD19 phycoerythrin (PE) or APC, CD10 FITC, CD36 FITC, CD20 PE, SmIgM PE, SmIgD FITC, CyIgM FITC, TdT FITC, CyCD179a PE, and CyCD79a PE. Gate exclusion was performed to eliminate T-cell, myeloid cell, natural killer cell, and basophil contaminations. BM B-cell stages were then identified as pro-B ($CD22^+CD19^-CD36^-$), pre-B1 ($CD19^+CD22^+CD10^+TdT^+CyIgM^-CD20^-$), pre-B2 ($CD19^+CD22^+CD10^+TdT^+CyIgM^+CD79a^+VpreB^+CD20^+$), and immature B ($CD19^+CD22^+CD10^+CD20^+CD79a^+SmIgM^+SmIgD^-$) cells, correcting the percentage of different subpopulations by exclusion of PB B-cell contamination. Calculations were performed as previously reported.[E5] Ten thousand events were acquired in the B-cell gate on a BD FACSCanto II (BD Biosciences, San Jose, Calif) and analyzed with DIVA software version 6.0.

Fifty microliters of whole blood was stained for B-cell subsets with the following mix of antibodies: CD19 PeCy7, CD24 FITC, CD38 PerCP5.5, BAFF-R PE, and CD27 APC (all from BD PharMingen). IgG and IgA FITC were purchased from Jackson Immunoresearch (West Grove, Pa). The blood was lysed for 10 minutes at room temperature, washed in PBS-FACS, and stained directly with antibody mix. In the case of IgG and IgA staining, a previous incubation with medium containing 10% FBS was performed. After staining and washing, the cells were fixed with 150 μL of PBS-FACS plus 0.2% formaldehyde and read within 24 hours. Thirty thousand events were registered in the lymphogate by using a BD FACSCanto II and analyzed with FlowJo software 2.2 (TreeStar, Ashland, Ore).

Reference values for all stainings were obtained from pediatric and adult donors selected with respect to the age of the patients enrolled in the study.[E6] Donors with less than 5% B cells were excluded from the analysis. Samples were acquired within 24 hours on a BD FACSCanto II and analyzed with FlowJo software 2.2.

For the isolation of precursor and naive B cells by means of FACS sorting, thawed samples were stained with CD34 PE, CD19 PeCy7, CD10 FITC, and IgM APC (all from BD PharMingen). The subpopulations were sorted with a BD FACSVantage Cell Sorter and tested by using qPCR for their level of gene correction.

BAFF ELISA

BAFF was evaluated by means of ELISA (R&D Systems, Minneapolis, Minn), according to the manufacturer's protocol, with 50 μL of plasma samples from patients and control subjects. BAFF levels were determined within 30 minutes with a microplate reader (Bio-Rad Laboratories, Richmond, Calif) set to 450 nm.

Proliferation assay

$CD20^+$ B cells from patients and donors were purified from frozen PBMCs thawed in RPMI and 1% FBS plus 10 μL/mL DNAse (Calbiochem, San Diego, Calif). Purity was greater than 74%. $CD20^+$ B cells were labeled with 0.5 μmol/L CFSE for 8 minutes at room temperature. CFSE-stained B cells (6×10^4) from patients undergoing HSC-GT and donors were plated in 96-well flat-bottom culture plates and stimulated for 72 hours in RPMI 10% Hyclone (Sera; Thermo Scientific, Uppsala, Sweden) with 2.5 μg/mL CpG2006 (5′-TsCsg sTsGsg sTsTsT sTsgsT sCsgsT sTsTsT sgsTsC sgsTsT-3′; TIB BioMol, Genoa, Italy), 2.5 μg/mL $F(ab')_2$ anti-human IgM/IgG/IgA (Jackson Immunoresearch), and 3 ng/mL CD40L (Alexis Biochemicals, San Diego, Calif). For patients undergoing ERT, 10×10^4 CFSE-stained B cells were plated. Cell culture was performed in a final volume of 200 μL. The group of control subjects is composed of pediatric and adult donors who were verified to proliferate in the same way after stimulation. Proliferating B cells were stained with anti-CD19 PeCy7 and 7-amino-actinomycin D to exclude dead cells and IgG and IgA APC at the third day of stimulation. A BD FACSCanto II was used to acquire 10,000 events for each condition in the lymphocyte gate. The results were analyzed with FlowJo software 2.2.

ELISpot

Plasmablasts secreting IgM were detected by using a spot ELISA, as previously described.[E7] Two thousand five hundred stimulated B cells per condition were analyzed, and the spots were counted with an A.EL.VIS Elispot Reader and software.

Statistical analyses

Normality assumption was checked, and parametric or nonparametric tests were applied accordingly to fulfillment of normality assumptions. A 2-tailed Mann-Whitney *U* test was used to assess whether the means of 2 independent groups were statistically different. Data were analyzed with GraphPad Prism, version 4.02 (GraphPad Software, La Jolla, Calif). To analyze longitudinal data, LME models were applied[E8] to elucidate patients' specific unobservable variability/heterogeneity by including random components. LME models were performed with R statistical software (version 2.15.3, http://www.R-project.org). A P value of less than .05 was considered significant. One-way ANOVA with the *post hoc* Bonferroni multiple comparison test was performed to assess the significance of differences between means of more than 2 independent groups. When the normality assumption was not met, the Kruskal-Wallis test with Dunn multiple comparison was applied.

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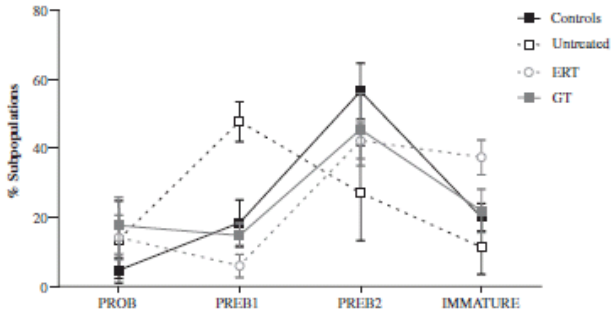

FIG E1. BM B-cell development in patients with untreated and treated ADA-SCID. Summary of BM B-cell development in healthy donors (n = 10, *black solid line*), patients with untreated ADA-SCID (n = 7, *black dashed line*), patients undergoing ERT (n = 6, *gray dashed line*), and patients undergoing HSC-GT (n = 8, *gray solid line*).

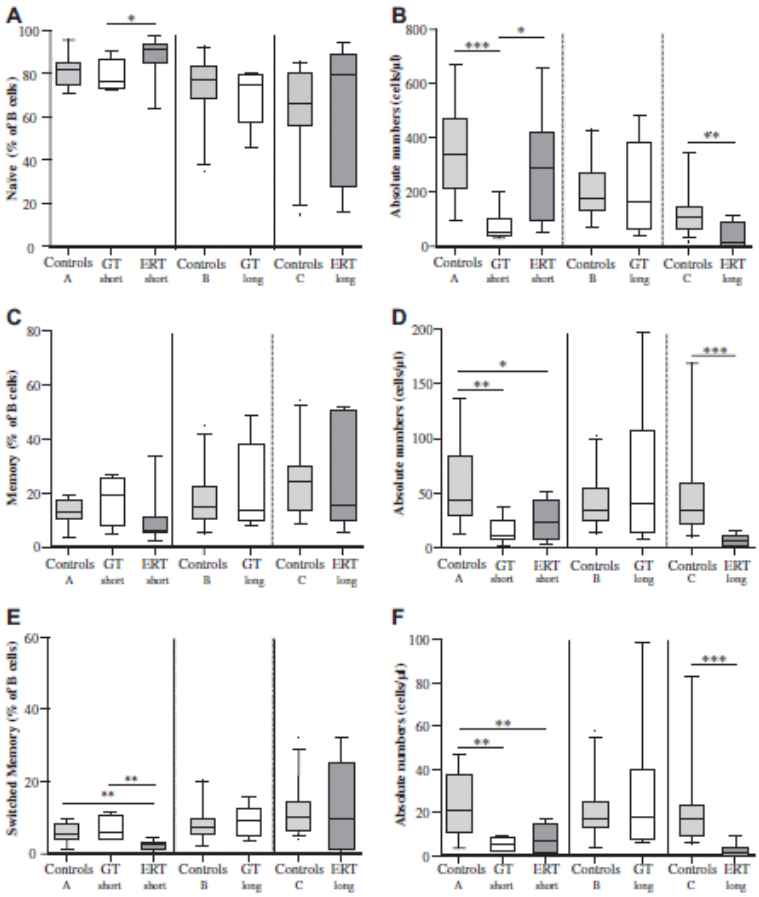

FIG E2. B-cell reconstitution in patients with ADA-SCID after different treatments. **A, C,** and **E,** Percentage of naive and memory B cells in patients after different treatments compared with their control reference. Data are presented as medians with 5th and 95th percentiles. **P* < .05 and ***P* < .005, Mann-Whitney test. **B, D,** and **F,** Absolute numbers of naive, memory, and switched memory B cells in the same groups. Data are presented as medians with 5th and 95th percentiles. **P* < .05, ***P* < .005, and ****P* < .001. Fig E2, *A-D*: Short-term HSC-GT (n = 8), short-term ERT (n = 8), long-term HSC-GT (n = 6), and long-term ERT (n = 6). Age-matched control subjects: Controls A (n = 14, 0.5-4 years), Controls B (n = 23, 4.1-13 years), and Controls C (n = 30, 13-25 years). Fig E2, *E* and *F*: Short-term HSC-GT (n = 4), short-term ERT (n = 8), long-term HSC-GT (n = 6), and long-term ERT (n = 6).

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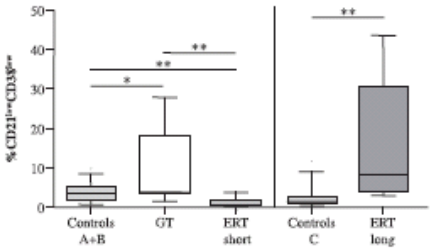

FIG E3. Determining $CD21^{low}CD38^{low}$ B-cell counts in patients. The presence of $CD21^{low}CD38^{low}$ B cells in the blood of 11 patients undergoing HSC-GT, 6 patients undergoing short-term ERT, and 5 patients undergoing long-term ERT compared with Controls A+B (0.6-13 years, n = 38) and Controls C (13-25 years, n = 31). Box and whiskers plots represent 5th and 95th percentiles. *$P < .05$ and **$P < .005$, Mann-Whitney test.

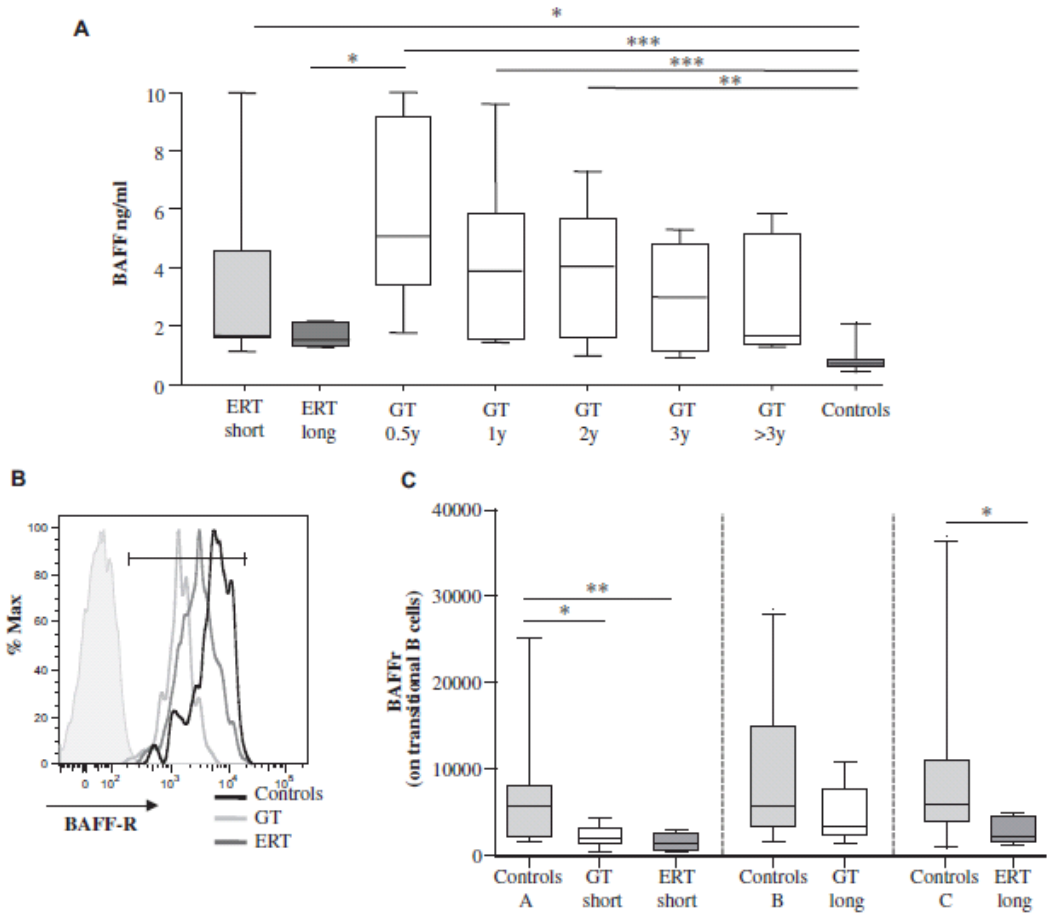

FIG E4. BAFF levels in plasma and BAFF-R expression on transitional B cells. **A,** Plasma levels of BAFF determined by means of ELISA in patients undergoing short-term ERT (n = 12), long-term ERT (n = 6), and HSC-GT (n = 11 for 0.5-3 years and n = 7 for >3 years). Data are compared with those of age-matched control subjects (n = 24). Data are presented as medians with 5th and 95th percentiles. *$P < .05$, **$P < .005$, and ***$P < .001$ 1-way ANOVA with Bonferroni test. **B,** Representative BAFF-R histograms on transitional B cells for patients undergoing HSC-GT *(gray line)*, patients undergoing ERT *(dark gray line)*, and control subjects *(black line)*. The *gray filled histogram* is isotype control. **C,** Mean fluorescence intensity of BAFF-R for patients undergoing short-term HSC-GT (n = 6), patients undergoing short-term ERT (n = 6), patients undergoing long-term HSC-GT (n = 5), and patients undergoing long-term ERT (n = 5) compared with control subjects. Age-matched control subjects: Controls A (n = 14, 0.5-4 years), Controls B (n = 21, 4.1-13 years), Controls C (n = 26, 13-25 years). Data are presented as medians with 5th and 95th percentiles. *$P < .05$ and **$P < .005$, Mann-Whitney test.

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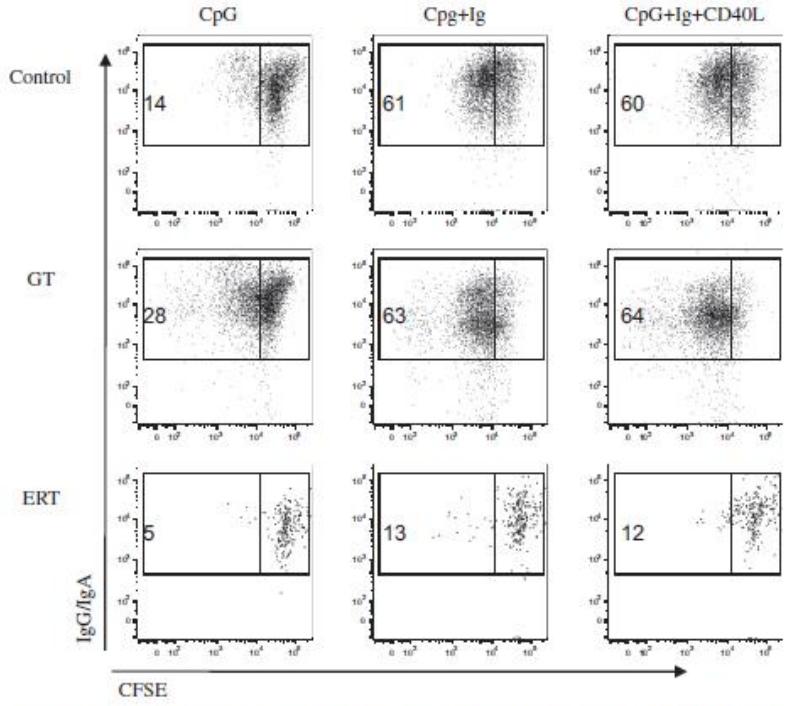

FIG E5. Gating strategy for B-cell proliferation. Percentage of IgG/IgA diluting CFSE after stimulation with CpG plus immunoglobulin or CD40L in representative patients and control subjects.

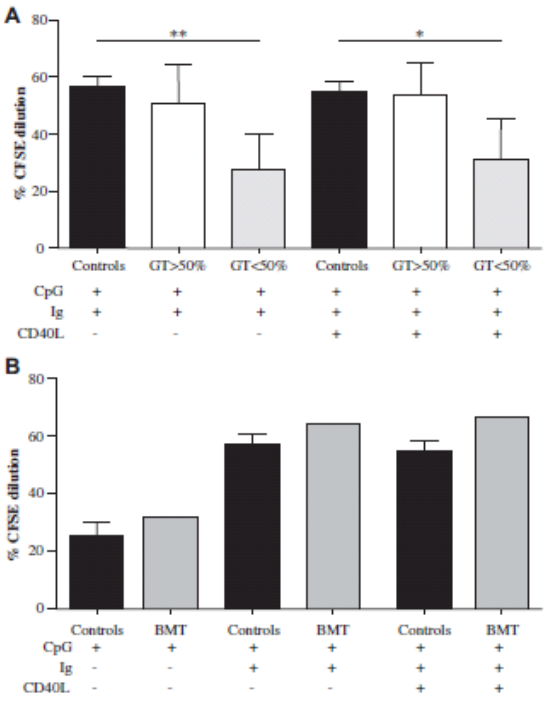

FIG E6. B-cell proliferation is dependent on level of ADA expression. **A,** Decreased B-cell proliferation after stimulation with CpG, immunoglobulin, and/or CD40L in patients undergoing HSC-GT with less than 50% transduced B cells (n = 3, <50%) compared with 3 patients undergoing HSC-GT with greater than 50% transduced B cells and 15 healthy donors. Data are presented as means ± SEMs. $*P < .05$ and $**P < .005$, Student *t* test. **B,** Normalization of B-cell proliferation in 1 BMT-treated patient compared with the same control subjects.

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TABLE E1. Characteristics of patients analyzed for BM B-cell development

Patient group	No. of patients	Age at analysis (y), median (range)	Years of follow up, median (range)
ADA-SCID	7	0.41 (0.13-13.5)	—
ERT	6	1.5 (0.5-5.6)	1 (0.2-5.3)
HSC-GT	8	5.4 (2.3-7.6)	3.9 (1-4.5)*
Control subjects	10	3.6 (1.3-5.1)	—

*Median percentage of gene-corrected BM B cells was 8.9% (range, 0.6% to 32.4%).

TABLE E2. Characteristics of patients analyzed for PB B-cell development

Patient group	No. of patients	Age (y)	Age of FU (y)	T-cell counts (no. of cells/μL)	Patients with normal T-cell counts*	B-cell counts (no. of cells/μL)	Patients with normal B-cell counts*	IVIg discontinuation and vaccination response†	Autoimmune manifestations
Short-term HSC-GT	8	4.8 (3.2-9.3)	3.1 (1-4)	650 (453-1929)	2 (25%)	72 (39-570)	1 (12.5%)	4 (50%)	Hypothyroidism, Guillain-Barrée syndrome§
Long-term HSC-GT	6	9.9 (6.2-12.1)	8 (4.6-8.9)	1487 (550-4412)	5 (83.3%)	160 (55-631)	2 (33.3%)	6 (100%)	—
Short-term ERT	8	1.9 (0.5-2.7)	1.1 (0.4-2.5)	1076 (28-4599)	5 (62.5%)	320 (65-724)	0	0‡	Hemolytic anemia‖
Long-term ERT	6	14.7 (9.2-24.6)	14.4 (9.1-22.8)	476 (70-1369)	2 (33.3%)	28 (4-120)	0	6 (100%)	TID, hemolytic anemia

Values for age and counts are presented as medians and ranges.
*Values are compared with those of healthy control subjects, as published by Comans-Bitter et al.[39] Numbers in parentheses indicate the percentage of patients with normal values.
†To vaccination (tetanus/pneumococcus).
‡One patient was not available because immunoglobulin levels were normal before ERT therapy.
§At the time of analysis, 2 of 14 patients had autoimmunity, and 1 additional patient had autoimmunity later.[31]
‖At the time of analysis, 1 of 8 patients showed mild hemolytic anemia.

Gene Therapy

Recent advances in gene therapy for primary immunodeficiencies

M.P. Cicalese[1]
F. Barzaghi[1]
I. Brigida[2]
M.C. Castiello[2]
G. Ottaviano[1]
R. Bacchetta[2]
A. Aiuti[2,3]
M.G. Roncarolo[1,2,4]

[1]Pediatric Immunohematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan, Italy
[2]San Raffaele Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute, Milan, Italy
[3]Department of Systems Medicine, Tor Vergata University, Rome, Italy
[4]Vita-Salute San Raffaele University, Milan, Italy

Correspondence:
Maria Grazia Roncarolo
E-mail: roncarolo.mariagrazia@hsr.it

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A B S T R A C T

Gene therapy with hematopoietic stem cells (HSC) is an attractive therapeutic strategy for primary immunodeficiencies (PIDs). In the last decade, there has been convincing evidence of long-term disease correction as a result of *ex vivo* viral vector-mediated gene transfer into autologous hematopoietic stem cells. Gene therapy with gammaretroviral vectors was shown to be efficacious for severe combined immundeficiencies as (SCID)-X1, adenosine deaminase (ADA)-deficient SCID, Wiskott-Aldrich Syndrome (WAS) and, with transient benefit, chronic granulomatous disease (CGD). The success of these early studies has been counterbalanced by the development of vector-related insertional mutagenic events in clinical trials for SCID-X1, WAS and CGD. Self-inactivating lentiviral vectors represent a promising strategy with improved safety and efficacy. Recently, gene therapy for WAS using HSC transduced with lentiviral vectors resulted in highly efficient gene corrected cells, restored immune functions and clinical improvement. Pre-clinical studies of FOXP3 gene transfer in IPEX have already shown encouraging results. If long-term efficacy and safety will be confirmed, gene therapy will become a standard treatment option for specific forms of PIDs and other monogenic diseases.

Learning goals

At the conclusion of this activity, participants should be able to:
- to identify the rationale for the treatment of specific PIDs with gene-therapy strategy and the pros and cons of this approach in comparison to allogeneic bone marrow transplantation;
- to analyze the safety issues related to the transduction of stem cells, the factors involved and the current approaches to overcome these issues.

Introduction

Primary immunodeficiencies (PIDs) constitute a heterogeneous group of conditions that result from mutations in over 300 different genes and are characterized by varying degrees of abnormal immune cell development and/or function.[1] The incidence of PIDs varies from 1 in 600 to 1 in 500,000 live newborns, depending upon the specific disorder.[2,3] The spectrum of disorders is rapidly advancing as next generation sequencing technologies become available and clinical awareness grows. Patients with PIDs display phenotypes that can range from being asymptomatic to manifestation of life-threatening conditions (e.g. various forms of severe combined immunodeficiency, SCID). Additionally, an increasing number of syndromes are also characterized by immune dysregulation with autoimmunity and susceptibility to lymphoreticular malignancy.[3-5]

While differing in clinical severity, early diagnosis and treatment is of considerable importance for all forms of PID to prevent organ damage and life-threatening infections. Much effort is currently being put into developing methods for detection of PIDs in the neonatal period, especially for PIDs with lack of functional T or B lymphocytes. PCR-based detection of signal joint T-cell receptor excision circles (TRECs) has proven to be a valuable tool for identifying patients with severe combined immunodeficiencies. Universal newborn screening has helped to establish the true incidence of SCID in California (1 in 66,250 live births) and has led to the improvement of survival outcome.[6] A similar method for analysis of k-deleting excision circles (KRECs) has been described for detection of patients with X-linked agammaglobulinemia (XLA). Recently, a robust triplex PCR method for quantitation of TRECs and KRECs, using a single Guthrie card punch, has developed and validated in a cohort of 2560 anonymized newborn screening cards. Through this method patients with SCID, XLA, ataxia-telangiectasia and Nijmegen-breakage-syndrome have been readily identified and effective newborn screening for severe immunodeficiency syndromes characterized by the absence of T or B cells has been made easier.[7]

Research activity in PIDs continues to produce new information on genes affecting the differentiation and function of cells of the immune system and new pathogenic mutations and molecular mechanisms have been reported. With improved access to gene sequencing, different clinical presentations are attributed to gene defects that, in the past, appeared to have a traditional presentation only. Despite the sig-

nal advance afforded by genotype analysis, patients with the same genetic mutations are found to express different phenotypes, leading the immunologists to look for other factors, both genetic and non-genetic, that regulate the gene expression.[6] Bone marrow transplantation is still the standard of care for patients with PIDs. In the largest cohort study on the outcome of patients undergoing hematopoietic stem cells transplantation (HSCT) for PIDs from European centers with the longest follow up, survival continues to improve over time. In patients with SCID, survival with genoidentical donors from 2000 to 2005 has been 90%. Survival using a mismatched relative has improved (66%), similar to that using an unrelated donor (69%). For younger patients with no pre-existing infection, such as newborns with SCID, the outcome is even better, demonstrating the usefulness of newborn screening programs in facilitating the early diagnosis of SCID and the advantage in performing transplantation before six months of age. Thus, survival can be expected to improve in the future.[8] For non-SCID PIDs, in the period from 2000 to 2005, transplantation from an unrelated donor has given a 3-year survival rate of 79%, similar to a genoidentical donor, while the outcome has been 46% in mismatched related donor transplants.[8] For patients without a suitable donor, autologous transplantation of genetically corrected hematopoietic stem and progenitor cells offers a life-saving alternative.[3,9-11] Current approaches are based on *ex vivo* transfer of therapeutic transgene via viral vectors to patient-derived autologous HSC followed by transplantation back to the patient with or without conditioning.[3,10-13]

It was thought that SCID-X1, the most frequent form of SCID, was the most accurate model for assessing gene therapy (GT), because spontaneous reversion of the mutation in the γc-retroviral IL2RG gene led to significant correction of the immune deficiency, supporting the hypothesis whereby transduced lymphocyte progenitors carry a selective advantage over their non-transduced counterparts. Between 1999 and 2006, 20 subjects with SCID-X1 were treated in 2 trials in Paris and then London, showing 85% survival and full or nearly full correction of the T-cell immunodeficiency.[10] Nevertheless, 5 out of 20 subjects have developed T-cell leukemia[10] between two and five years after GT; 4 of them have been into remission after chemotherapy, while the remaining patient has died from chemotherapy-refractory leukemia. In all cases, the adverse event was the result of insertional oncogenesis due to aberrant expression of the LMO2 or CCND2 oncogenes induced by the integration of the γc retroviral vector in the proximity of the gene regulatory regions.[14] The occurrence of these serious complications prompted discontinuation of these trials.[10]

An additional trial begun at the NIH in 2003 to offer a rescue treatment option for older X-SCID patients who had failed to respond to HCT. Three patients (11, 10 and 14 years old) were enrolled in this trial that used a γ-retroviral vector and targeted G-CSF-mobilized peripheral blood $CD34^+$ cells. T-cell numbers and function significantly improved in the youngest subject, but no immunological improvement was observed in the other 2 subjects, perhaps due to age-dependent loss of thymic function.[14]

Among the SCID forms, the T-B-SCID due to Artemis gene mutation is another excellent candidate for GT. For these patients, it is reported an increased post-transplantation morbidity, characterized by auto-inflammatory complications, severe and persistent graft-*versus*-host disease (GvHD), recurrent and severe infections for an unsatisfying immunological reconstitution, due to the defect in DNA repair mechanisms. Pre-clinical models have demonstrated the biological efficacy of GT approach and safety studies are ongoing to complete pre-clinical evaluation and move to clinical application.[10]

HSCT for chronic granulomatous disease (CGD) has recently shown a high success rate as an early intervention in patients with very low superoxide production and in patients with a history of invasive fungal infection, liver abscesses and/or significant inflammatory or autoimmune signs.[11] This constitutes an argument in favor of the GT approach for patients without a matched donor. Most recent trials for X-CGD conducted in 5 different centers worldwide (Frankfurt, Zurich, London, NIH, Seoul) in combination with reduced intensity conditioning, resulted in initial higher correction of NADPH activity and transient clinical benefit in some patients.[11] The current experience with GT of CGD points to a yet unexplained difficulty in achieving long-term engraftment of significant levels of transduced cells. The lack of a strong selective advantage of gene-corrected populations in this disease may play a major role and may indicate that more significant levels of HSC transduction and engraftment will be needed to obtain clinical benefit.[14] On the other hand, the 1st-generation γ-retroviral vectors used in these protocols have also been associated with a high incidence of severe adverse events in the patients with persistent gene marking. A myelodysplastic syndrome (MDS) occurred in 3 patients (2 in Frankfurt, with fatal outcome, and 1 in Zurich). The second child treated in Zurich displayed a clonal expansion without monosomy 7 or MDS and this clone disappeared after a successful early HSCT.[11]

Apart from PIDs, in the last two decades significant advances have been made in GT for hemoglobinopathies, as beta (β)-thalassemia and sickle cell disease. Clinical trials with β-/γ- globin lentivirus vectors are now open at multiple sites and transfusion independence following GT has been reported in one patient with β-thalassemia.[15]

Metabolic diseases have been another recent field of application of GT, with the proof of successful ABCD1 gene transfer to autologous hematopoietic stem cells by a lentiviral vector derived from HIV-1 in 3 patients with X-linked adrenoleukodystrophy.[16] The first clinical trial of GT with lentiviral vector with ARSA gene showed extensive and stable ARSA gene replacement in the first 3 presymptomatic treated patients with metachromatic leukodystrophy (MLD); moreover, the disease did not manifest or progress in those patients 7 to 21 months beyond the predicted age of symptom onset.[17]

Here we provide an overview of our experience in gene therapy approaches targeting ADA-SCID, WAS and IPEX and the future perspectives of gene therapy for PIDs.

ADA-SCID gene therapy

Mutations in the Adenosine Deaminase (ADA) gene, a purine salvage enzyme that catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine into inosine and 2'-deoxyinosine respectively, are the cause of 15-20% of all cases of SCIDs.[18,19] Affected children accumu-

late the toxic metabolites in plasma, lymphoid tissues and red blood cells, resulting in severe lymphopenia (T, B and NK and absence of cellular and humoral immune function.[20] In addition, non-immunological alterations have been described, as organ damage, neurological and skeletal alterations.[21,22] In the absence of treatment, the condition is fatal in the first year of life.

Bone marrow transplantation (BMT) from an HLA-identical sibling donor is the standard treatment for ADA-SCID. Most patients survive and achieve complete immune recovery, although the procedure remains still available for a minority of patients.[19,23] Enzyme replacement therapy (ERT) with PEG-ADA was introduced as a lifesaving, non-curative treatment : for patients lacking a HLA-matched donor,[24,25] aiming at decreasing toxic metabolites concentrations, thereby correcting the metabolic abnormalities. A variable extent of immune recovery was observed, often associated with an incomplete long-term immune recovery, a gradual decline to mitogenic proliferative response and to antigenic responses a few years after treatment, lymphopenia and requirement of IVIg replacement.[26,27] Furthermore, patients under long-term treatment display loss of regulatory T-cell function, impaired B-cell development, immune dysregulations, development of antibodies against bovine ADA and autoimmune manifestations, including hemolytic anemia, and immune thrombocytopenia.[26,28-35]

Gene therapy with retrovirally transduced HSC has been developed as a successful and safe alternative strategy for those ADA-SCID patients that could not have access to BMT or for whom ERT was not sufficient to maintain adequate immune reconstitution.

Since 2000, over 40 patients have been enrolled in clinical trials with reduced intensity conditioning in Italy, UK, and USA, resulting in long-term multilineage engraftment, sustained systemic detoxification and improved immune functions.[12,33,36,37]

Eighteen patients have been treated at San Raffaele Telethon Institute for Gene Therapy, Milan, in pilot studies and GT clinical trial[12] (A Aiuti, unpublished data, 2014) and on compassionate use program (A Aiuti, unpublished data, 2014), receiving a reduced intensity conditioning regimen with busulfan (4 mg/kg) after discontinuing PEG-ADA in order to facilitate the selective advantage for gene-corrected cells[12,44] (A Aiuti, unpublished data, 2014). At present, treated children are alive, and only 3 out of 18 patients have required ERT or BMT after gene therapy[12] (A Aiuti, unpublished data, 2014). Gene corrected cells were detected in all myeloid and lymphoid subsets, the latter being more represented due to their survival advantage. The reconstitution of the immune system was also documented by the recovery of polyclonal thymopoiesis in the majority of patients, with normalization of the Vbeta repertoire, increase of T-cell counts and improvement in their TREC levels during follow up. Similar results were obtained by the groups of Great Hormond Street Hospital,[36] Children's Hospital Los Angeles (CHLA) and Clinical Center of the National Institutes of Health (NIH),[37] demonstrating the clinical efficacy of ADA gene transfer in restoring normal immune and metabolic functions in the majority of ADA-SCID patients treated. Furthermore, the study by Candotti *et al*. compared patients treated with or without chemotherapy confirming the importance of pre-conditioning on the engraftment of myeloid cells and immune reconstitution.[37]

The progressive restoration of immune and metabolic functions led to significant improvement of patients' development and protection from severe infections, without adverse events related to gene therapy related to GT. However, non-transduced cells co-existed with transduced T, B and NK cells in the periphery, suggesting a possible rescue of non-transduced cells by the detoxification provided by gene-corrected cells. The presence of shared vector integrations among multiple hematopoietic lineages demonstrated stable engraftment of multipotent HSC. Integrations were also found within and/or near potentially oncogenic loci, but did not result in selection or expansion of malignant cell clones *in vivo*[38-40] underlining the importance of a continuous monitoring of the safety of this treatment. The development of innovative vector technology, such as lentiviral vectors, might further improve the vector integration safety profile. The first vector developed was a self-inactivating (SIN)-lentiviral vector driving ADA expression from the phosphoglycerate kinase (PGK) promoter.[41] Mice treated with GT early in life were rescued from their lethal phenotype and displayed adequate immune reconstitution and metabolic correction. A comparative approach between different treatment options revealed important information on their efficacy and established a model for autoimmunity in the context of long-term PEG-ADA treatment.[33] Carbonaro *et al*. recently tested a novel self-inactivating lentiviral vector with a codon-optimized human cADA gene under the control of the short form elongation factor-1α promoter to maximize the expression and biological activity of the transgene. The engineered cells showed efficient ADA gene transfer into murine HSC, with ability to restore ADA expression, and induce immune recovery of T and B cells and good thymic reconstitution.[42] These studies led to 2 clinical trials to test the *in vivo* safety and efficacy of lentiviral-mediated gene therapy (*clinicaltrials.gov identifier: 02022696, 01852071*).

WAS gene therapy

Wiskott-Aldrich Syndrome (WAS) is a complex X-linked primary immunodeficiency that affects 1-10 out of a million male individuals. Clinical manifestations of the disease are microthrombocytopenia, high susceptibility to infections, eczema and increased risk of autoimmune manifestations and tumors.[43] The gene responsible for WAS (*WAS* gene) is placed on the X chromosome[44] and encodes a 502 amino acid protein (Wiskott-Aldrich Syndrome protein, or WASp), involved in actin polymerization[45] and other important signaling activities.[46,47] Thus, absence or residual WASp expression causes functional defects in immune cells, of both innate and adaptive immune system, and this makes WAS a very complex immunodeficiency. The life expectancy of WAS patients is severely reduced, unless they are successfully cured by BMT.[48] Unfortunately, a significant fraction of patients lack a suitable HLA-matched donor. In addition, transplanted patients with a lower degree of chimerism show an incomplete reconstitution of lymphocyte counts and a high incidence of autoimmunity.[48,49] For these reasons, therapy with WAS gene-corrected autologous HSCs could represent a valid alternative approach.

Extensive pre-clinical studies have been performed in the last 15 years to evaluate the feasibility and efficacy of gene transfer by means of both γ-retroviral (RV) and lentiviral (LV) vectors. The availability of *Was*$^{-/-}$ mice has been crucial in accurately evaluating short- and long-term safety and efficacy of gene therapy (GT *in vivo*). Gene transfer with Moloney-derived γ-RVs effectively restored protein expression and corrected the defects in cytoskeletal organization, cellular activation and inflammation both in *in vitro* and in *in vivo* models of WAS.[50-54] Because LVs offer a better safety profile as compared to RVs,[55] we and others started to develop a gene transfer approach based on a LV encoding the human WASp cDNA under the control of the human *WAS* endogenous promoter.[56,57] The safety and efficacy of LVs were demonstrated both in progenitor[58-60] and mature hematopoietic cells of WAS patients.[57,59,61] Gene therapy with LV-transduced *Was*$^{-/-}$ HSCs in *Was*$^{-/-}$ mice did not cause any adverse events or tumors even in long-term follow-up studies[62] and led to robust transduction and restoration of WASp expression and immunological functions in T, B and dendritic cells.[62-64] In addition, human CD34^{+} cells LV-transduced and transplanted in immunodeficient mice displayed a normal engraftment and differentiation ability.[60] A recent study directly compared SIN-LVs containing γ-retrovirus-derived promoter and the human proximal WASp endogenous promoter. Results indicate that the γ-retrovirus-derived promoter lead to a stronger transgene expression as compared to the WAS-promoter vector but this occurs in association with myeloid clonal expansion and transcriptional dysregulation, highlighting the potential risk of the use of strong viral promoter.[65]

The encouraging results of pre-clinical studies settled the basis for conducting GT clinical trial for WAS. A first phase I/II study was conducted since 2007 in Hannover, including 10 patients, treated with WASp-expressing LTR-driven γ-RV following myelosuppressive conditioning.[66] The first report, describing the 2.5-year follow up of 2 WAS patients treated with this approach, provided the proof of principle of *ex vivo* HSPCs GT for WAS. Stable engraftment of gene-corrected cells in multiple lineages (HSCs, lymphoid and myeloid cells) lead to restoration of WASp expression. As previously observed in mixed chimerism pre-clinical models,[67] a clear proliferative and selective advantage of corrected lymphoid cells over myeloid lineage was also evident in clinical context. Functional improvement of B and T cells, as well as monocytes and NK cells, occurred in both patients, providing the correction of immunological defects of WASp deficiency, and platelet counts increased to safer levels after GT. These results were confirmed in a larger cohort of patients, who showed partial to complete resolution of immunodeficiency, autoimmunity, and bleeding.[68,69] The analysis of vector common insertion sites (CISs) revealed a marked clustering between patients, with hotspots found within the proto-oncogenes (LMO2 and MDS/Evi1), already known to be associated in other GT trials with the development of leukemia and myelodysplasia.[68,70-72] This high-precision clustering observed by integration analyses suggests that *in vivo* clonal selection of the affected clones can account for this phenomenon. Between 16 months and 5 years after GT, 7 out of 10 treated patients developed hematologic malignancies.[68,69] These included 4 cases of T-cell acute lymphoblastic leukemia (T-ALL), 2 primary T-ALL with secondary AML and one acute myeloid leukemia (AML). Vector integration analyses revealed dominant clones with insertions at the LMO2 loci (6 T-ALL), MDS1 (2 AML), or MN1 (1 AML locus). Six out of 7 patients who developed malignancy are alive after treatment with conventional chemotherapy or allogeneic stem cell transplantation (alloSCT), but one patient died due to progressive leukemia after alloSCT.[68] These data indicate that LMO2-driven leukemogenesis is not specific for γc-SCID GT, but it is also seen in WAS GT. The vector configuration, which is based on a strong viral promoter in the context of a RV, the high number of RV copies contained in CD34^{+} cells, and the disease background might have contributed to the high frequency of malignancy. The use of self-inactivating LV with improved safety profile containing a cellular promoter could overcome this safety issues.

GT clinical trials based on LVs have started in Europe and the US in the last few years, using different conditioning regimens and enrolling patients with severe clinical score and without a suitable BMT donor.[3] In the San Raffaele Telethon Institute for Gene Therapy, Milan, 6 patients have been treated in the GT clinical trial since 2010, and the data on the first 3 patients have been recently reported.[13] Patients were treated with autologous genetically-corrected HSCs preceded by a reduced intensity conditioning with busulfan and fludarabine. Cells were transduced with a LV encoding the human WASp cDNA under the control of the human *WAS* endogenous promoter. All patients showed a multilineage engraftment of corrected cells, both in bone marrow and peripheral blood compartment, with stable levels of WASp expression in cells after 2.5 years from GT. The immunological function restoration involved T- and B-cell compartment, as well as the cytotoxic activity of NK cells and the suppressive activity of Tregs. Furthermore, platelet counts appeared to be increased with respect to the pre-GT values and platelets presented with normal volume. These biological improvements led to a clinical benefit for all treated patients, with the reduction of severity and frequency of infections, and the absence of autoimmune manifestations. Moreover, WAS patients did not experience high-grade bleeding events after GT (Figure 1A). Interestingly, the levels of corrected cells in the bone marrow appeared significantly higher than the engraftment levels achieved in the RVs trial, suggesting that LV-GT provides a higher gene transfer efficiency with repopulating HSCs. In addition, this study provided a relevant comparison between RV-GT and LV-GT for WAS in terms of safety. Analysis of LVs insertion profile *in vivo*, showed that LVs integrations are less prone to cluster near to genes involved in hematopoietic functions and potential proto-oncogenes, as occurred in RV-GT trial (Figure 1B and C). Moreover, highly represented genes targeted by the vector in these WAS patients were also hit in other LV-GT trials,[16,17] where no clonal expansion or leukemia have been reported. Finally, LVs showed a classical distribution within transcriptional units and gene-rich regions, while RVs integration were more commonly observed close to transcription start sites. This evidence confirms that a SIN configuration and the presence of an autologous WASp human promoter allow a safer GT approach for WAS, minimizing the risk of clonal expansions.

A longer evaluation of all 6 patients enrolled in our trial,

together with data of other LV-GT trials, will be important to confirm that GT is a safe and effective treatment and should be preferred to other therapeutic options when a full HLA-matched donor is not available.

Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare monogenic primary immunodeficiency. Typical presenting symptoms are severe enteritis and/or type 1 diabetes mellitus, alone or in combination with eczema and elevated serum IgE. The disease has usually a severe early onset and can become rapidly fatal within the first year of life regardless of the type and site of the mutation. Other autoimmune symptoms, such as hypothyroidism, cytopenia, hepatitis, nephropathy, arthritis, and alopecia, can develop in patients who survive the initial acute phase.[73,74] Recently, some atypical cases, with delayed onset and low autoimmune burden, have been reported.

The current therapeutic options for IPEX patients are limited. Early intervention is required to control symptoms at onset. However, supportive and replacement therapies combined with pharmacological immunosuppression allows only a reduction of the autoimmune manifestations but cannot cure the disease. The only known curative therapy for IPEX syndrome is BMT, but it is always limited by the availability of a suitable donor and the lack of specific guidelines in the context of this disease.

This severe form of monogenic autoimmunity is due to mutations on the *FOXP3* gene, responsible for severe impairment of naturally occurring (n) regulatory T cells (Tregs). FOXP3 is a transcription factor, which exerts a key role in the function of nTreg cells.[75-79] These cells are among the main subsets of CD4$^+$ T cells aimed at maintaining peripheral self-tolerance.

In IPEX patients, *FOXP3*mut Tregs are present but functionally impaired, determining lack of peripheral tolerance and consequent autoimmune manifestations.[80-82]

Functional *in vitro* studies on Tregs of IPEX patients revealed that the degree of suppressive activity impairment varies among patients with different mutations. The suppressive function is absent in patients with null mutations.[80] The Tregs-mediated suppression mechanisms remain controversial and the impact of different *FOXP3* mutations on Tregs function remains unknown.

Moreover, it has recently been described that *FOXP3* mutations cause high instability of Tregs, making FOXP3 mutated cells prone to lose their suppressive function and to convert from a regulatory to an effector (i.e. Th17) phenotype. Becoming IL-17-producing cells, these cells may directly contribute to the autoimmune damage in the target organs in the presence of an inflammatory environment.[83] Even if the impairment of Tregs function is the major pathogenetic event in IPEX syndrome, other factors, such as inflammation, Th17 elevation, T-effector cell dysfunction and altered cytokine production,[80,84,85] can contribute to maintaining the immune-dysregulation. Data from healthy carriers of *FOXP3* mutations and transplanted IPEX patients with low peripheral donor chimerism clearly indicate that Tregs expressing a wild-type FOXP3 survive long term in the periphery displaying a selective advantage. Moreover, the presence of a FOXP3 wild-type Tregs subpopulation is sufficient to maintain peripheral tolerance.[86,87] Thus, the administration of functional Tregs in IPEX patients could allow efficient suppression of the pathogenic effector counterpart and could contribute to control the overwhelming autoimmunity.

Infusion of *ex vivo* expanded Tregs has been already used to prevent graft-*versus*-host disease to prevent GvHD in patients after HSCT. Results are promising even though the experience is limited.[88-91] This strategy was shown to be potentially helpful also to inhibit rejection in solid organ transplantation, and controlling autoimmunity in patients with T1D.[92]

With the aim of developing a gene therapy approach, Tregs have been obtained *in vitro* by gene transfer of wild-type FOXP3, demonstrating that ectopic overexpression of FOXP3 in conventional CD4$^+$ T cells from healthy donors can produce Tregs-like cells, with low proliferative potential, reduced cytokine production and potent suppressive function *in vitro*,[93-95] but also *in vivo*, in a human-

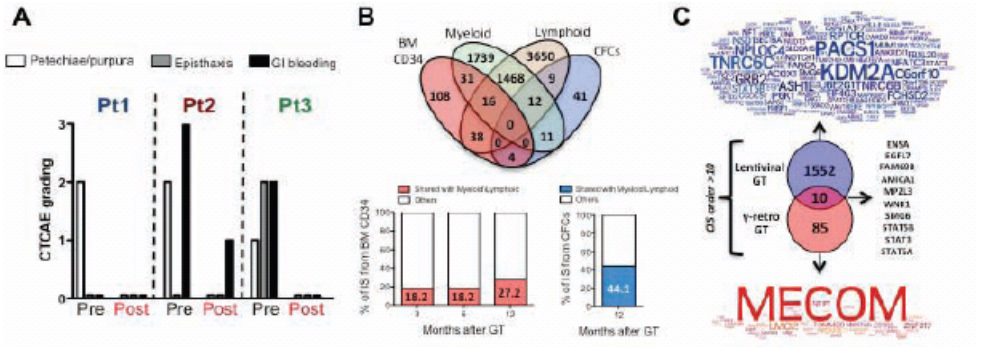

Figure 1. Lentiviral-mediated hematopoietic stem cell gene therapy for Wiskott-Aldrich Syndrome. (A. Grading of bleeding events in WAS patients (pre- and post-GT). (B. Venn diagram: shared insertions among different lineages and clonogenic progenitors (CFC). Columns: fraction of shared insertions in CD34$^+$ cells and CFCs. (C. Common insertion sites in lentiviral versus γ-retroviral GT trials (adapted from Aiuti *et al.*[13]).

ized murine model of xenogeneic GvHD (i.e. immunodeficient mice previously injected with allogeneic human CD4+ T cells to induce GvHD).[96] After injection of FOXP3-transduced cells, mice showed improved survival and reduced weight loss, indicating a clinical improvement of the GvHD. Importantly FOXP3-transduced cells alone did not expand *in vivo* and did not induce GvHD, maintained high expression of Tregs markers (such as CD25 and CTLA4) and produced a low amount of cytokine, despite the presence of an inflammatory environment. Similarly, CD4+ cells from patients with IPEX syndrome have been efficiently transduced by the same lentiviral vector carrying wild-type FOXP3 and they not only acquired a Tregs-like phenotype (expressing high CD25 and CTLA4, with low CD127, in addition to several Tregs lineage molecules) but also drastically reduced their cytokine production and acquired Tregs-like functional properties (suppressive activity and anergy). Moreover, these corrected cells protected mice from lethal xenogeneic GvHD (improving survival) and allowed better maintenance of the weight.[96]

These observations pave the way for the treatment of IPEX patients by adoptive cell therapy with genetically engineered Tregs. This approach could restore tolerance in patients with IPEX (or other autoimmune disorders of different origin), allowing maintenance of long-term immune modulation and avoiding general immunosuppression and systemic toxicity.

Conclusions

There is great expectation in the patients' community that safe and effective approaches can be developed for a wider spectrum of PID and other genetic diseases. Advances in molecular genetics, vector design and HSC biology are favoring the extension of clinical trials to several PID variants. Pre-clinical experiments are ongoing for a number of PID (Artemis deficiency, CD3γ deficiency, JAK3-SCID, LAD-1, PNP deficiency, RAG1/2 deficiency, X-HIM, XLA, XLP, ZAP70 deficiency) that would benefit from gene therapy approaches.[14] Several of such diseases represent significant challenges and will require additional progress beyond the current available technologies.

On the other hand, BMT has become much safer and more successful over time, and the outcome of transplantation from matched unrelated donor has improved in the last 15 years mostly for non-SCID PID,[8] so gene therapy has to be carefully considered in the view of its potential risks and benefits. Variables such as the choice of vector, the patient's inflammatory status, bone marrow stem cell reservoir and the ability to transduce a high number of HSCs need to be evaluated more thoroughly to significantly improve the anticipated advantage of gene therapy over allogeneic transplantation for non-SCID patients. The use of the conditioning regimen has been identified as a crucial factor in achieving therapeutic levels of gene corrected, multilineage HSC, leading to superior engraftment in the myeloid lineages in the ADA-SCID as compared to the SCID-X1 trial. This finding has important implications for translating the experience of PID to other blood borne disorders, such as lysosomal storage disorders and thalassemia, which require a higher therapeutic threshold. To address the issue of lymphoid or myeloid proliferation[10,11] associated with insertional mutagenesis observed in SCID-X1, CGD and WAS clinical trials, the γ-retroviral vectors are being increasingly replaced by self-inactivating by SIN vectors, which have shown high efficacy in terms of sustainable transgene expression and reduced risk of insertional mutagenesis tendency for harmful mutagenesis *in vitro*[97] and *in vivo*.[13,17]

Ultimately, a general shift from the current 'gene addition' approaches to 'gene editing' strategies will be required and some of these efforts have already been tested on PID models, such as the early demonstrations of the IL2RG gene repair with zinc-finger nuclease technology[14] and the more recent development of site-directed gene addition strategies for CGD in induced pluripotent stem cells.[14] The current successes of GT show that, even if still distant from randomized studies, a new knowledge is going to be added to conventional BMT, and this new targeted medicine will be further developed in the near future.

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Human Gene Therapy

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TITLE PAGE

Invited Review

Title: Clinical applications of gene therapy for Primary Immunodeficiencies

Authors: Maria Pia Cicalese (MD) [1,2] and Alessandro Aiuti (MD, PhD) [1,2,3]

Affiliations:

[1] San Raffaele Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute Milan, Italy;

[2] Pediatric Immunohematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan;

[3] Vita-Salute San Raffaele University, Milan, Italy;

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CORRESPONDING AUTHOR: Alessandro Aiuti, MD, PhD.

ADDRESS FOR CORRESPONDENCE: Via Olgettina, 60; 20132, Milan, Italy

Telephone Number Work: +390226434387; FAX +390226436545

Email: aiuti.alessandro@hsr.it

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Abstract

Primary Immunodeficiencies (PIDs) have represented a paradigmatic model for successes and pitfalls of haematopoietic stem cells gene therapy (HSC-GT). First clinical trials performed with gamma retroviral vectors (γ-RV) for adenosine deaminase deficiency (ADA-SCID), X-linked SCID (SCID-X1), and Wiskott-Aldrich Syndrome (WAS) showed that gene therapy is a valid therapeutic option in patients lacking a HLA-identical donor. No insertional mutagenesis events have been observed in > 40 ADA-SCID patients treated so far in the context of different clinical trials worldwide, suggesting a favorable risk benefit ratio for this disease. On the other hand, the occurrence of insertional oncogenesis in SCID-X1, WAS and CGD RV clinical trials, prompted the development of safer vector construct based on self-inactivating (SIN) retroviral or lentiviral vectors (LVVs). Here we will present the recent results of LVV mediated gene therapy for WAS showing stable multilineage engraftment leading to haematological and immunological improvement, and discuss the differences with respect to the WAS RV trial. We will also describe recent clinical results of SCID-X1 gene therapy with SIN γ-RV and the perspectives of targeted genome editing techniques, following early preclinical studies showing promising results in terms of specificity of gene correction. Finally, we provide an overview of the gene therapy approaches for other PIDs and discuss its prospects in relation to the evolving arena of allogeneic transplant.

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Introduction

Primary Immunodeficiencies (PIDs) represent a heterogeneous group of monogenic conditions determined by altered immune responses of either innate and/or adaptive immunity (1). More than 260 disorders have been identified, resulting from mutations in over 300 genes (2, 3). Their number is rapidly increasing thanks to next generation sequencing technologies and increased clinical awareness (2).

The incidence of PIDs varies from 1 in 600 to 1 in 500,000 live newborns, depending upon the specific disorder (4, 5). Patients with PIDs display phenotypes that can range from being asymptomatic to manifestation of life-threatening conditions (e.g. various forms of severe combined immunodeficiency, SCID). With new information on genes affecting the immune system and discovery of new pathogenic mutations and molecular mechanisms, different clinical presentations are attributed to gene defects that, in the past, appeared to have a traditional presentation only (2, 6).

Additionally, an increasing number of syndromes are also characterized by immune dysregulation with autoimmunity and susceptibility to lymphoreticular malignancy (5, 7, 8).

While differing in clinical severity, early diagnosis and treatment remain a mainstay for all forms of PID to prevent organ damage and life-threatening infections and to improve prognosis and quality of life (6, 9). Major efforts have recently been undertaken to develop methods for detection of PID in the neonatal period, in particular a triplex RT-qPCR measuring the levels of TRECs and KRECs have been shown to provide a suitable screening for the vast majority of severe immunodeficiency diseases characterised by T- or B-lymphopenia in newborns (*6*). Universal newborn screening in US has helped to establish the true incidence of SCID in California (1 in 66,250 live births) and has led to the improvement of survival outcome (*9*). Recently, tandem mass spectrometry for analysis of metabolites from dried blood spots has been proposed as an easy and cheap method for ADA SCID screening (10, 11*)*.

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Bone marrow transplantation still remains the definitive cure for most of the PIDs and the outcomes of patients treated in European centres are improving over time (12, 13). Survival is excellent in HLA genoidentical donor setting and is progressively increasing in other settings thanks to the improvement in conditioning regimens, prophylaxis and treatment of infectious complications, GvHD prevention, stem cells selection and manipulation and choice of unrelated donors (13-17).

In the last 15 years, gene therapy has been successfully implemented for the treatment of PID patients who lacked a suitable donor. In some cases, efficacy of gene therapy has been counterbalanced by the occurrence of insertional oncogenesis. The understanding of the molecular events that led to oncogenesis and improved vector technology, allowed to progress with safer gene therapy approaches for PID.

Here we will review the successfully most recent results on clinical trials for X-linked, ADA SCID, WAS and CGD and discuss perspectives for new technologies and other diseases.

Gene therapy for SCID-X1

X-linked SCID is actually the most common form of SCID, accounting for 40%–50% of SCID cases reported worldwide (*5*). Mutations in the IL2RG gene are leading to defective expression of the common gammachain (γc), a subunit shared by a host of cytokine receptors including interleukin (IL)-2, 4, 7, 9, 15 and 21 receptor complexes, which play a vital role in lymphocyte development and function (*5*). As a consequence, SCID-X1 patients present profound immunological defects caused by low numbers or complete absence of T and NK cells, and presence of non-functional B cells (18). Death from community-acquired or opportunistic infections usually occurs before 1 year of age unless allogeneic hematopoietic stem cell transplantation is performed (19).

While allogeneic transplantation from HLA-identical donor has a high survival rate, persistent defects in humoral or cellular functions have been reported for some patients, resulting in partial

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immune recovery, autoimmunity and/or retarded growth (19, 20). On the other hand, transplantation from mismatched related, matched unrelated, or umbilical cord donors in patients with ongoing infection is associated with lower survival rates, often partial chimerism of hematopoietic lineages with persistent impairment of humoral immune function (21), and higher rates of complications as graft-versus-host disease (12-14, 21).

SCID-X1 was thought to be the most accurate model for assessing GT, because spontaneous reversion of the mutation in the γc-encoding IL2RG gene led to restore of immunological competence, suggesting that transduced lymphocyte progenitors could carry a selective advantage over their non-transduced counterparts (5, 22).

Between 1999 and 2006, 20 subjects with SCID-X1 lacking HLA identical bone marrow donors have been treated in 2 trials, conducted in Paris and London. The treatment consisted in an infusion of autologous CD34+ bone marrow cells transduced with a first-generation Moloney murine leukemia virus vector expressing the γc complementary DNA (MFG-γc) and containing duplicated viral enhancer sequences within the long terminal repeats (LTRs). Gene therapy resulted in correction of the immunodeficiency, with polyclonal and functional T-lymphocytes in 19/20 patients (3, 23). Engraftment and correction of NK and B cells was lower, likely because patients did not receive conditioning. Immunoglobulin replacement treatment was stopped in 11/20 patients, allow in most patients to live a normal life (3, 23). A 85% survival was observed with a median follow-up of 13 years, similar to the results obtained with matched-sibling donor HSCT (19), demonstrating that gene therapy can be curative for X-SCID with long-lasting (10 years) beneficial effects (19). Four patients in the French trial and 1 patient in the British cohort have developed T-cell leukemia (24) between 2 and 5 years after GT: 4 of them have been into remission after conventional chemotherapy, in one case followed by matched unrelated HCT, and remain in long-term remission (25, 26), while the remaining patient has died from chemotherapy-refractory leukemia. In all cases, the adverse event was the result of insertional oncogenesis due to aberrant expression of the LMO2 (LIM domain only- 2) or CCND2 (cyclin D2) oncogenes induced by the integration of the γc retroviral vector in the proximity of the gene regulatory regions

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(19). Second genome alterations were found in all cases and probably accounted for the advent of overt leukemia (3), favoured by the selective advantage conferred to them by the concomitant expression of the γc gene (23).

The occurrence of these serious complications prompted discontinuation of these trials (24).

A further trial was started at the NIH in 2003 as a treatment option for older X-SCID patients for whom HCT was not successful. Three patients (11, 10 and 14 years old) were treated with G-CSF-mobilized peripheral blood CD34+ cells transduced with γ-retroviral vector. An improvement of T-cell numbers and function only in one subject, the youngest, and no immunological improvement in the other 2 (23).

To improve safety while maintaining the efficacy profile for X-SCID gene therapy, a self-inactivating (SIN) γ-retroviral vector with deleted Moloney murine leukemia virus LTR U3 enhancer was exploited, expressing the IL2RG complementary DNA from the eukaryotic human elongation factor 1α (EF1α) short promoter, and having shown to be less mutagenic in vitro, although effective in the mouse model of X-SCID (enhancer-deleted SIN-γc) (27, 28). The interim results of the first 9 patients treated in parallel phase 1/2 trials conducted in London, Paris, Boston, Cincinnati, and Los Angeles have been recently published (19) and the trial is still recruiting patients **(Table)**. SCID-X1 children were enrolled if a HLA identical sibling donor was not available or in case of severe ongoing, therapy-resistant, infections. Eight out of nine treated patients survived, while a preexisting disseminated adenovirus infection was fatal to one patient 4 months after GT, before the full reconstitution of the T-cell compartment. Up to 48 months of follow-up, immune reconstitution of T-cells occurred in other 7 patients and was comparable to that observed in the previous trials conducted in Paris and London. Importantly, integration analysis showed a polyclonal integration profile with reduced numbers of clones near known lymphoid proto-oncogenes and genes implicated in serious adverse events in previous GT trials (19).

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A new approach based on the use of self-inactivating lentiviral vector (CL20-i4-EF1α-hγc-OPT) expressing a γc gene has been developed by Sorrentino and colleagues (29) and is used in a two-site clinical trial. Typical X-SCID patients will be enrolled at the St.Jude Children's Research Center in Memphis (ClinicalTrials.gov identifier: NCT01512888), while atypical children and adolescents between 2 and 20 years of age are treated at NIH (NCT01306019) **(Table)**. The latter arm of the trial uses nonmyeloablative conditioning with a total busulfan dose of 6 mg/kg/body weight to improve the efficacy of engraftment of gene-corrected cells (30). Preliminary results on in two young adults after 15 and 9 months from GT show restoration of Ig production and B-cell function, increasing gene marked NK cells, and clinical improvement (*Suk See De Ravin*, ASGCT 2014, personal communication).

Gene therapy for ADA-SCID

ADA SCID, caused by mutations in the ADA gene impairing ADA activity, stability, and survival and leading to accumulation of toxic metabolites in plasma, red blood cells and tissues, represents the second most frequent form of SCIDs, accounting for 15-20% of all cases of severe combined immunodeficiencies (31, 32). In its typical early severe onset form, it is usually fatal in the first year of life (33). Apart from the profound lymphopenia (T, B and NK) and the absence of cellular and humoral immune function (34), non-immunological alterations as manifestation of the metabolic organ damage have been described (35, 36).

HLA-matched sibling (MSD) or family donor (MFD) SCT is the gold standard in ADA-SCID therapy and is associated to excellent overall survival (86% for MSD and 83% for MFD). Data from a large cohort of ADA SCID patients transplanted with alternative donors over 20 years, clearly show the importance of donor matching in improving outcome, with 67% OS in HLA-matched MUDs (67%) and 43% and 29% OS in haplo and mismatched unrelated donor SCT, respectively (37, 38). Moreover, the metabolic nature of the disease and the need for conditioning regimens makes mismatched transplantation for this form of SCID more difficult to manage than other forms, even

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for the associated risks (21, 32). Beyond this, once patients survive the procedure and engraft donor cells, relatively complete immune reconstitution is achieved (37).

Patients who receive enzyme replacement therapy (ERT) usually improve immune functions and are well detoxified, but on long-term present with T-cell numbers that are below normal levels and show gradual decline of functional assays, whereas B-cell function defects are not fully repaired, with only 50% of patients able to discontinue Ig replacement therapy (37). Moreover, a significant part of patients show immune dysregulations, development of antibodies against bovine ADA and autoimmune manifestations over time (39-46).

The first gene therapy attempts, aimed to provide treatment in patients lacking a HLA-identical sibling donor, started in the early 1990s, targeting T lymphocytes from patients on ERT (47, 48). Despite this approach was not sufficient to discontinue stably ERT, it was shown that the transduced T cells could safely persist for more than 10 years (49, 50). Importantly, a recently identified population of T cells with stem cell properties was shown to significantly contribute to the pool of long-term living T cells by tracking of insertion sites (50). Early attempts at gene therapy targeting CD34 progenitor cells were unsuccessful because of a low transduction rate and the decision to keep patients on ERT (51).

Ameliorations in transduction and protocols changes were introduced in the GT study designs were introduced in order to improve the engraftment of modified stem cells, as well as to provide a selective pressure for the corrected cells that would ultimately translate into clinical benefit for the patients (52). The engraftment of infused stem cells was optimized by the inclusion of a mild pre-conditioning regimen with Busulfan (4 mg/kg i.v.), to make space for the corrected progenitors (53). Finally, the selective pressure for outgrowth of gene-modified progeny was provided by the withdrawal of ERT prior to GT. A report on the results obtained at TIGET, Italy, on the first 10 patients showed in 8/10 patients ADA levels sufficient to gain decrease of toxic metabolites and allow functional immune recovery. Thymic activity was restored to normal with polyclonal T cell receptor repertoires. Normal serum immunoglobulin levels were detected in 50% of patients, allowing for discontinuation of immunoglobulin therapy and production of antibodies after immunization. Importantly, no leukemic or adverse events related to the therapy were observed

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(54). A recent update showed 18 patients treated with a F-U of > 1 year, who are all alive; among them, 15 are off-ERT **(Table)** (Cicalese MP, personal communication, ESID 2014).

Subsequently, other patients were treated in London and US with a slight different approach in terms of conditioning regimen and vector design (52) **(Table)**. As a result, in 31 out of 42 globally treated patients GT was efficacious, leading to the ERT discontinuation and persistent immune reconstitution, long-term multilineage engraftment and sustained systemic detoxification (3, 54-56, Cicalese MP, personal communication, ESID 2014; Shaw KL, ASGCT 2014, personal Communication; Carbonaro Sarracino D, ESID 2014, personal communication).

Furthermore, the study by Candotti et al. compared patients treated with or without chemotherapy confirming the importance of preconditioning on the engraftment of myeloid cells and immune reconstitution (56).

Importantly, the presence of shared vector integrations among multiple hematopoietic lineages demonstrated stable engraftment of multipotent HSC (57).

Differently from SCID X1 trial, and despite of the use of the same first-generation γRV vectors, there were no genotoxic events in GT-treated SCID patients. Integrations were also found in ADA SCID patients within and/or near potentially oncogenic loci, but did not result in selection or expansion of malignant cell clones in vivo (57, 58 and Aiuti, unpublished data), suggesting that ADA deficiency in itself may create an unfavorable milieu for leukemogenesis, perhaps by restricting cell proliferation at a critical stages of thymopoiesis. These results underline the importance of a continuous monitoring of the safety of this treatment.

Based on safety issues arisen in clinical trials of retroviral GT for the treatment of other PIDs, alternatively strategies based on ADA encoding lentiviruses (LVV) were developed. Mortellaro et al developed a SIN LVV in which the expression of the human ADA gene was driven by a PGK promoter. Mice treated with GT early in life were rescued from their lethal phenotype and displayed adequate immune reconstitution and metabolic correction, similar to bone marrow transplantation (59). To further improve ADA expression, the group of Dr. Kohn and Dr. Gaspar designed an LVV that included a codon-optimized human cADA gene under the control of the short form elongation factor-1α promoter (LV EFS ADA) that displayed high-efficiency gene transfer and adequate ADA

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expression to rescue ADA -/- mice from their lethal phenotype with good T- and B-cells reconstitution. An in vitro immortalization assays demonstrated that LV EFS ADA had significantly less transformation potential compared to gRV vectors, without clonal skewing (60).

On this basis, two phase I/II clinical trials with the use of LV EFS ADA are actually ongoing in UK and US (clinical trials NCT02022696, NCT01852071) for the treatment of ADA SCID children **(Table)**. To date, 5 patients aged between 1.2-4.5yrs have been treated, after conditioning with Busulfan i.v. at a single dose of ~5mg/kg. At a mean follow up of about 1 year, there has been significant immunological recovery, with a rise of total T cell and CD4+ counts and normalization in mitogen responses (Gaspar et al HB, personal communication, ASGCT 2014).

The promising results from gene therapy trials led to issue recommendation from the EBMT Inborn Error Working Party, according to which gene therapy is considered a valid option to all patients without an HLA-identical sibling donor, regardless of the age, availability of a MUD, and outcome of PEG-ADA therapy (61).

Gene therapy for WAS

Wiskott-Aldrich Syndrome (WAS) is a rare, complex, X-linked primary immunodeficiency disorder caused by mutations in the WAS gene (62) characterized by recurrent infections, microthrombocytopenia, eczema and increased risk of autoimmune manifestations and tumors (63). The prevalence is estimated to be 1 to 10 out of a million male individuals, with an incidence of 4 out of a million male live births. The WAS protein (WASp) is a key regulator of actin polymerization in haematopoietic cells (64), thus, absence or residual WASp expression causes functional defects in different leukocyte subsets, as defective function of T and B cells, alteration in NK cell immunological formation synapse and impaired migration of all leukocyte subsets (65, 66). The life expectancy of WAS patients is severely reduced, unless they are successfully cured by BMT (*15*). At present, HSCT from HLA-identical sibling donor (matched sibling donor, MSD) is the treatment

of choice for WAS, with a reported 82 to 88% long-term survival in different European and American centres in the past decade (*67-69*), with a survival close to 100% for patients transplanted after year 2000 *(15)*. MUD transplant has reported recently survivals of 85-90%, but better results are obtained when patients are transplanted before the age of 5, and autoimmune complications are more frequent when complete chimerism is not achieved (*67*). HSCT from alternative donors (partially HLA-matched relatives, or mismatched family donors – MMFD, and umbilical cord blood – UCB) has led to more disappointing results.

In this scenario, therapy with WAS gene-corrected autologous HSCs could represent a valid alternative approach for patients lacking a suitable donor or older than 5 years (70).

Extensive preclinical studies have been performed in the last 15 years to evaluate the feasibility and efficacy of gene transfer by means of both γ-retroviral (RV) (71-74) and lentiviral (LV) vectors (75-76).

Based on the encouraging results of preclinical studies, a first phase I/II study on humans was conducted since 2007 in Hannover, including 10 patients, treated with WASp-expressing LTR-driven γ-RV following reduced-intensity myeloablation (77-79). Stable engraftment of gene-corrected cells in multiple lineages (HSCs, lymphoid and myeloid cells) lead to restoration of WASp expression. As previously observed in mixed chimerism pre-clinical models (80) a clear proliferative and selective advantage of corrected lymphoid cells over myeloid lineage was also evident in patients. These results were confirmed in a larger cohort of patients, who showed partial to complete resolution of immunodeficiency, autoimmunity, and bleeding (77, 81). However, the analysis of vector common insertion sites (CISs) revealed a marked clustering between patients, with hotspots found within the proto-oncogenes (LMO2 and MDS/Evi1), already known to be associated in other GT trials with the development of leukemia and myelodysplasia (3, 77, 82, 83). Between 14 months and 5 years after GT, 7 out of 10 treated patients developed hematologic malignancies (77, 81). These included 4 cases of T-cell acute lymphoblastic leukemia (T-ALL), 2 primary T-ALL with secondary AML and one acute myeloid leukemia (AML), all LMO-2 related. Despite chemotherapy and secondary allogeneic HSCT, two patients died from leukemia (77).

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These data indicate that LMO2-driven leukemogenesis is not specific for γc-SCID GT, but it is also seen in WAS GT. The strong viral promoter in the context of a RV, the relatively high vector copy number per cell (1.7 to 5.2), and the disease background might have contributed to the increased risk of insertional mutagenesis (77).

While retroviral WAS gene therapy was still at preclinical level, alternative approaches with lentiviral vectors were developed to overcome the issues related to gammaretroviral vector (84). The own WAS promoter was chosen to drive WASp expression to reduce the risk of insertional oncogenesis and allow a more physiological expression of the transgene. Extensive preclinical studies showed the lack of toxicity in the mouse model of the disease (85). Moreover, human CD34+ cells were effectively transduced in vitro with the vector and engrafted in immunodeficient mice (86). Clinical trials were then started in Europe and the US **(Table)**, using different conditioning regimens and enrolling patients with severe clinical score and without a suitable BMT donor (5). Results of the first three patients have been recently published (87). After a reduced intensity conditioning with busulfan and fludarabine, patients received autologous HSCs, transduced with the LV encoding the human WASp cDNA. All patients showed a multilineage engraftment of corrected cells, both in bone marrow and peripheral blood compartment, with stable levels of WASp expression. The immunological function restoration involved T and B cells compartment, as well as cytotoxic activity of NK cells and suppressive activity of Treg. Furthermore, platelet counts increased with respect to the pre-GT phase, and platelets presented with normal volume. These biological improvements lead to a clinical benefit for all treated patients, with a reduction of severity and frequency of infections and bleeding and the absence of autoimmune manifestations. The levels of corrected cells in the bone marrow were significantly higher than the engraftment levels achieved in the previous RVs trial, suggesting a higher gene transfer efficiency of LV. In terms of safety, analysis of LVs insertion profile in vivo showed that in contrast to RV-GT, LVs integrations are less prone to cluster near genes involved in hematopoietic functions and potential proto-oncogenes. Moreover, highly represented genes targeted by the vector in these WAS patients were also hit in other LV-GT trials (88), where no clonal expansion or leukemia have been reported. A SIN-LV configuration and the presence of an autologous WASp human promoter

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in the vector construct were crucial in developing a safer GT approach for WAS (89). A longer evaluation of all patients treated, together with data of other LV-GT trials, will be important to confirm the safety and efficacy of this approach.

Another LV vector using a viral MND-derived promoter has also been used to further increase WASp expression in mice, but results indicate that the γ-retrovirus-derived promoter lead to a stronger transgene expression as compared to the WAS-promoter vector. Moreover, this occurs in association with myeloid clonal expansion and transcriptional dysregulation, highlighting the potential risk of the use of strong viral promoter (3, 90).

Gene therapy for CGD

Mutations impairing the expression of gp91phox, p22phox, p47pox, or p67phox molecules are affecting the superoxide production in phagocytic cells leading to CGD disorder, in which life-threatening abscesses and/or skin, liver, lung or bone granuloma, and inflammatory complications are characteristic (23, 91). Available therapeutic strategies include antibiotic long-life prophylaxis, IFN-γ administration and HCT (23, 92). HSCT has recently shown a high success rate as an early intervention in patients with very low superoxide production and in patients with a history of severe invasive fungal infection, organ abscesses and/or significant inflammatory or autoimmune signs (17, 93). This constitutes an argument in favor of the GT approach for patients without a matched donor. Early clinical trials performed with γRV without conditioning showed only transitory functional correction of ≤ 0.5 % of peripheral blood granulocytes (94, 95). Since gene transduced neutrophils have no survival advantage over defective neutrophils and have a lifespan of only a few days, engraftment of relatively high number of gene-transduced HSCs is required by preparatory conditioning (91).

Most recent trials for X-CGD were conducted in 5 different centers worldwide (Frankfurt, Zurich, London, NIH, Seoul) using γRV vector-transduced, mobilized CD34+ cells and non-myeloablative conditioning with low dose (8 to 10 mg/kg) busulfan (93, 96, 97, 98) ± fludarabine (92), or

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melphalan alone (140 mg/m2) (98) in more than 10 patients. The treatments resulted in initial transient improvement of functional neutrophils up to 30%, with clearance of severe fungal infections and clinical benefit, followed by a yet unexplained difficulty in achieving long-term engraftment of significant levels of transduced cells, with loss of the expression of the therapeutic gene gp91phox (99). The methylation of the viral promoter leading to silencing of transgene expression is an hypothesis suggested for loss of engraftment (83). Alternatively, ectopic gp91phox expression in HSPC could cause the production of reactive oxygen species that may damage DNA, alter cell growth, or induce apoptosis (100, 101, 102). Moreover, immune-mediated mechanisms against gp91phox expressing cells could have contributed to the lack of long-term persistence (102). On the other hand, the 1st-generation γ-retroviral vectors used in these protocols have also been associated with a high incidence of severe adverse events in the patients with persistent gene marking. A myelodysplastic syndrome (MDS) occurred in 3 patients (2 in Frankfurt, with fatal outcome, and 1 in Zurich). The second child treated in Zurich displayed a clonal expansion without monosomy 7 or MDS and this clone disappeared after a successful early HSCT (93). The frequency of these adverse events highlight the fact that only gp91phox-transduced cells with gain of function event could persist in patients treated with GT protocols employing LTR-driven RV (102).

All these events were associated with the insertion near MDS-EVI-1 protoncogenes, suggesting the necessity to improve the safety and the efficacy of gene transfer technology (83, 92, 97, 99, 103). At the same time, different strategies to restrict transgene expression to the mature phagocyte compartment were developed using SIN lentiviral vectors and have been tested in preclinical and clinical development **(Table)**. A gp91phox-encoding vector driven by synthetic chimeric promoter in combination with different myeloid transcription factors binding sites or the A2UCOE element linked to a myeloid promoter driving gp91phox expression in murine myeloid cells (102, 104, 105, 106). However, as A2UCOE protects from promoter methylation, its chromatin remodelling properties could have considerable side-effects in HSCs (105,107), so further studies are needed to proceed to clinical applications (108).

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Another recent approach to improve and maximize transgene expression in differentiated myeloid cells and minimizing its effect in HSCs is aiming to use a miR-126 target sequence fused to the transgene driven by a myeloid-specific promoter was proposed. In these vectors, transgene expression provided by the myeloid-specific promoter in myeloid cells and stringent control of gp91phox expression by the miR-126 target sequence in HSCs supports the further development of this microRNA approach as an alternative gene transfer technique for CGD (102,106).

A multicentric trial in collaboration between UK, Switzeland and Germany using a lentiviral vector with the chimeric promoter was approved and is currently recruiting (ClinicalTrials.gov [internet]. Bethesda (MD): National Library of Medicine (US). https://clinicaltrials.gov/. [Accessed March 2015];109) **(Table),** also if preliminary results suggest that gp91phox expression was detected only short-term during follow-up (98; Thrasher, ESGCT 2013, personal communication). Preclinical studies for the above dual-regulated lentiviral vector gene therapy approach are currently ongoing (109).

New Technologies and Future Plans

The success of gene therapy achieved in the last years has been the result of improved technology and enlarged knowledge on PID and their molecular mechanisms.

As the safety of the patients remains a crucial point, the use of new generation vectors, as SIN vectors or LVs, showing high efficacy in terms of sustainable transgene expression and reduced risk of insertional mutagenesis tendency *in vitro* and *in vivo*, has been preferred for certain PIDs, characterized by an increased risk of oncogenesis for their genetic background.

Progresses in vector design and HSC biology are favoring the extension of clinical trials to several PID variants, particularly to some challenging ones, for which the current available technologies are not sufficient. Pre-clinical experiments are ongoing for PID, such as Artemis deficiency, CD3γ deficiency, JAK3-SCID, LAD-1, PNP deficiency, RAG1/2 deficiency, X-HIM, XLA, XLP, ZAP70 deficiency, IPEX, that would benefit from gene therapy approaches (23).

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Furthermore, these results have now been translated from PIDs to other blood borne disorders, such as lysosomal storage disorders, (β)-thalassemia and sickle cell disease, which require a higher therapeutic threshold. Clinical trials with β-/γ- globin lentivirus vectors are now open at multiple sites and transfusion independence following GT has been reported in one patient with β-thalassemia (110). In the metabolic diseases field, gene therapy has led to successful ABCD1 gene transfer by lentiviral vector in autologous engineered cells of patients with X-linked Adrenoleukodystrophy (111) and stable LV ARSA gene replacement in patients with metachromatic leukodystrophy (MLD) (88).

On the other hand, BMT has become much safer and more successful over time for PID patients, thanks to early diagnosis, also due to newborn screening programs, and to the improved outcome of transplant from matched unrelated donor determined by new conditioning regimens, accuracy of typing and new cells manipulation processes. The long-term benefits, safety and cost-effectiveness of gene therapy vs allogeneic BMT should be evaluated thoroughly in the next years, together with practical issues, as the choice of vector, the patient's bone marrow stem cell reservoir and the manufacturing ability to transduce a high number of HSCs.

Gene editing will represent a further step to provide a correction in the defective genes at their genomic locus, maintaining appropriate regulatory control of gene expression and reducing the risk of genotoxicity through ectopic vector insertion. Zinc finger nucleases (ZFNs), meganucleases (MN), transcription activator-like effector nucleases (TALENS) and, more recently, clustered, regularly interspaced, short palindromic repeat (CRISPR) nucleases are all being developed to create highly specific gene targeting (112, 113, 114, 115, 116). The efficiency of gene editing using these techniques has been shown in cell lines and certain primary cell lineages, although remains limited in primary HSC (91).

In conclusion, gene therapy for PID is quickly moving from being an experimental approach to a standard cellular therapy, as demonstrated by the adoption of vector manufacture by mainstream pharmaceutical companies, on the basis of the encouraging results. Further refinement and standardization of the technology will be important for the future clinical development and enter into the arena of approved therapies.

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Authors' Disclosure Statement

Dr. Maria Pia Cicalese and Prof. Alessandro Aiuti declare that no competing financial interests do exist.

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Table. Ongoing Gene Therapy Clinical Trials for PID.

Disease	Country	Sponsor	Vector	Outcome	Reference	Clinical Trials.gov ref#
SCID-X1	France UK US	Assistance Publique - Hôpitaux de Paris, Great Ormond Street Hospital, Children's Hospital Boston	SIN γ-RV	8/9 patients alive, immune recovery with clinical benefit.	Hacein Abina et al., NEJM 2014	NCT01410019 NCT01175239 NCT01129544
SCID-X1	US	St. Jude Children's Research Hospital, National Institute of Allergy and Infectious Diseases,	SIN-LV	Two young adult patients, improved IgG production	De Ravin et al., ASGCT 2014	NCT01512888 NCT01306019
ADA-SCID	Italy	GlaxoSmithKline	γ-RV	15/18 patients off ERT, clinical benefit	Aiuti et al., NEJM 2009; Cicalese et al., J Clin Immunol (2014) 34 (Suppl 2):S311	NCT00598481
ADA-SCID	UK	Great Ormond Street Hospital	γ-RV	4/6 patients off ERT, clinical benefit.	Gaspar et al., Mol Ther 2006; Gaspar et al. Science Transl Med 2011.	NCT01279720
ADA-SCID	USA	Donald B. Kohn, UCLA	γ-RV	9/10 patients off ERT, clinical benefit.	Candotti et al., Blood 2012; Carbonaro Sarracino et al., J Clin Immunol (2014) 34 (Suppl 2):S313	NCT00794508
ADA-SCID	UK US	Great Ormond Street Hospital; Donald B. Kohn, UCLA	SIN-LV	5 patients, immune and metabolic recovery	Gaspar et al. , J Clin Immunol (2014) 34 (Suppl 2):S167	NCT01380990 NCT01852071
WAS	Italy	IRCCS San Raffaele	SIN-LV	6 patients, immune, hematological and clinical improvement ,	Aiuti et al, Science 2013; Aiuti et al., Hum Gene Ther (2014) 25:A7	NCT01515462
WAS	France UK	Genethon	SIN-LV	France: 4/5 patients	Bosticardo et al., Curr Gene	NCT01347346 NCT01347242

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				alive, clinical improvement US	Ther. 2014	
WAS	US	Children's Hospital Boston	SIN-LV	2 patients, immune and hematological improvement	Williams, Hum Gene Ther (2014) 25:A16	NCT01410825
CGD	Germany	Hubert Serve, Johann Wolfgang Goethe University Hospitals	SIN γ-RV			NCT01906541
CGD	Germany Switzerland UK US	Genethon UCLA	SIN-LV		Kauffmann, EMBO Mol Med 2013	NCT01855685 NCT02234934

Footnotes. Studies classified as active, ongoing, based on information retrieved from ClinicalTrials.gov. Updated information on recruitment status can be found on ClinicalTrials.gov.

Chapter 2

“Cell Therapy of Duchenne Muscolar Dystrophy by intra-arterial delivery of HLA-identical allogeneic mesoangioblasts”

original article

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Inflammation Converts Human Mesoangioblasts Into Targets of Alloreactive Immune Responses: Implications for Allogeneic Cell Therapy of DMD

Maddalena Noviello[1], Francesco Saverio Tedesco[2], Attilio Bondanza[1], Rossana Tonlorenzi[3], Maria Rosaria Carbone[1], Mattia Francesco Maria Gerli[2], Sarah Marktel[4], Sara Napolitano[4], Maria Pia Cicalese[4], Fabio Ciceri[4], Giuseppe Peretti[5], Giulio Cossu[2,4,6] and Chiara Bonini[1]

[1]Experimental Hematology Unit, Division of Immunology, Transplantation and Infectious Diseases, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; [2]Department of Cell and Developmental Biology, University College London, London, UK; [3]Institute of Experimental Neurology (INSPE), San Raffaele Scientific Institute, Milan, Italy; [4]Hematology Clinical Unit, Division of Regenerative Medicine, Gene Therapy and Stem Cells, San Raffaele Scientific Institute, Milan, Italy; [5]Regenerative Orthopedic Department, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy; [6]Institute of Inflammation and Repair, University of Manchester, Manchester, UK

Stem cell therapy is a promising approach to regenerate healthy tissues starting from a limited amount of self-renewing cells. Immunological rejection of cell therapy products might represent a major limitation. In this study, we investigated the immunological functional profile of mesoangioblasts, vessel-associated myogenic stem cells, currently tested in a phase 1–2a trial, active in our Institute, for the treatment of Duchenne muscular dystrophy. We report that in resting conditions, human mesoangioblasts are poorly immunogenic, inefficient in promoting the expansion of alloreactive T cells and intrinsically resistant to T-cell killing. However, upon exposure to interferon-γ or differentiation into myotubes, mesoangioblasts acquire the ability to promote the expansion of alloreactive T cells and acquire sensitivity to T-cell killing. Resistance of mesoangioblasts to T-cell killing is largely due to the expression of the intracellular serine protease inhibitor-9 and represents a relevant mechanism of stem cell immune evasion.

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INTRODUCTION

Stem cell therapy relies on the ability of a limited number of stem cells to engraft, self-renew, and properly differentiate when transplanted into patients affected by degenerative diseases. On top of these demanding requirements, stem cells need to evade the recipient immune system. When genetically corrected autologous cells are used, vectors and transgenes become putative targets of an immunological rejection.[1–3] Whereas, in the allogeneic setting, a plethora of antigens might render cells highly immunogenic. Alloreactive T-cell responses can be directed against unshared human leukocyte antigen (HLA) molecules or against minor histocompatibility antigens (mHAgs), peptides derived from polymorphic intracellular proteins presented in the context of HLA. An additional level of complexity is added by the pathological condition to be treated that is often associated to inflammation, a *milieu* that favors neutralizing immune responses. Such responses might result in the elimination of donor cells, thus reducing or even vanishing the therapeutic effort. On the other hand, several reports suggest that stem cells are unique in their ability to elude and modulate immune responses.[4,5] In our Institute, a cell therapy protocol is running to treat Duchenne muscular dystrophy (DMD) with the infusion of human pericyte-derived mesoangioblasts (MAB) harvested from healthy HLA-identical siblings. DMD is an X-linked recessive disease caused by mutations of the dystrophin gene and subsequent absence of the encoded sarcolemma protein. DMD is the most common and one of the most severe forms of muscular dystrophies. In DMD patients, primary wasting of skeletal and cardiac muscle leads to progressive loss of motility, respiratory, and cardiac failure and to premature death. Although restoration of dystrophin expression is the main goal to cure DMD, immune intervention has also been proposed to control inflammatory and possibly immune mechanisms secondary to fiber degeneration.[6] A cDNA microarray analysis of skeletal muscles from presymptomatic DMD patients revealed a molecular signature dominated by inflammatory responses, extracellular matrix remodeling, and muscle regeneration.[7] In addition to the local inflammation documented by immune cell infiltrates in damaged muscle, inflammatory mediators, such as interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) have been detected at high levels in muscles[8] and in plasma of DMD patients, suggesting a systemic inflammatory state.[9] The most compelling evidence of the pathological role of inflammation and immune dysregulation in DMD is the observation that anti-inflammatory compounds partially ameliorate disease course.[10] Nevertheless DMD remains an incurable disease and several experimental strategies have been developed over the last few years, including mutation-specific treatments to repair the endogenous gene and gene and cell therapy approaches to replace the mutated gene and/or affected cells.[11] Among the mutation-specific treatments, the exon-skipping strategy is designed to restore a disrupted open reading frame in an effort to produce

Correspondence: Chiara Bonini, Experimental Hematology Unit, Division of Regenerative Medicine, Gene Therapy and Stem Cells, S. Raffaele Scientific Institute, DIBIT Via Olgettina, 58 20132 Milan, Italy. E-mail: bonini.chiara@hsr.it

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a shortened but functional dystrophin and to recover a milder phenotype. In two clinical trials, >30 patients were systematically injected with splice-switching oligomers. New dystrophin expression was observed in muscle fibers but clinical improvement was modest, bringing into question the minimal amount required and the functionality of the produced dystrophin.[12,13] The identification of different types of mesoderm stem/progenitor cells opened new perspectives in the treatment of DMD. In particular, MAB represent a population of stem cells, able to differentiate in myotubes *in vitro* and *in vivo*, and able to produce functional improvements when injected intra-arterially in preclinical models of DMD.[14–17] Therefore, gene-modified autologous or healthy allogeneic MAB represent promising sources for cell/gene therapy of DMD. Unfortunately, host immune reactions against viral vectors,[18] dystrophin,[19] or other antigens expressed by transferred cells may limit the clinical outcome. To predict the risk of rejection, we analyzed the immunological profile, the immune stimulatory, and antigen presentation abilities of MAB in resting conditions and after treatment with IFN-γ to mimic the inflammatory *milieu* encountered in dystrophic muscles.

RESULTS

IFN-γ treatment does not alter the lineage expression profile of MAB

To verify the immunological profile of human MAB, MAB were isolated from muscle biopsies of 14 healthy donors, age ranging between 22 and 70 years, as previously described.[16] MAB were expanded *in vitro* and analyzed before passage XV to avoid senescence.[16,20] We observed alkaline phosphatase activity and high expression of CD44, CD146, CD13, and CD49b pericyte markers on cultured cells. The absence of the CD56 myoblast marker, CD117 hematopoietic marker, CD45 leukocyte marker, and CD31 endothelial marker confirmed the MAB nature of cultured cells (Figure 1a). To mimic the inflammatory conditions that frequently complicate muscles diseases, and that might alter the immunological properties of MAB, we exposed MAB to 500 IU/ml of the IFN-γ proinflammatory cytokine for 48 hours. We observed that IFN-γ treatment does not alter the lineage expression profile of MAB (Figure 1b).

Human MAB display a basal hypoimmunogenic phenotypic profile that can be partially reverted in an inflammatory *milieu*

The immunological profile of resting MAB and MAB exposed to IFN-γ (MABγ) was analyzed. Besides the interaction between T-cell receptor on T lymphocytes and the HLA–peptide complex on target cells, costimulatory receptor triggering and cell–cell interactions through adhesion molecule engagement are critical elements for T-cell activation. The expression of several molecules involved in the immunological synapse was quantified by fluorescence-activated cell sorting (FACS) analysis. Relative fluorescence intensity (RFI) was measured as the ratio of the mean fluorescence intensity of specific markers to the mean fluorescence intensity of isotype controls. In resting conditions, MAB express HLA

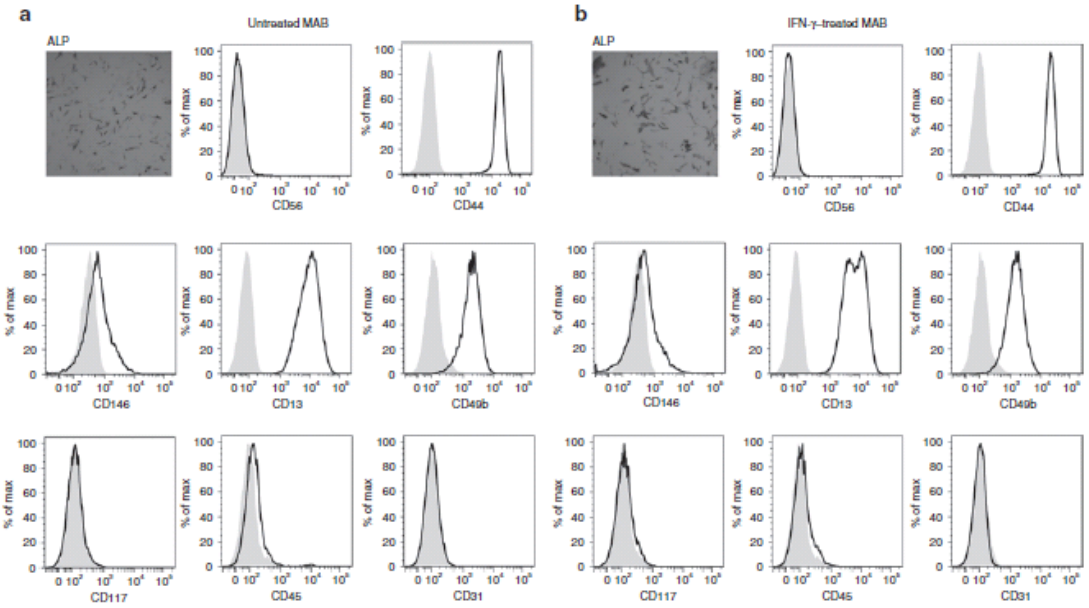

Figure 1 Interferon-γ (IFN-γ) treatment does not alter the lineage expression profile of mesoangioblasts (MAB). Human MAB obtained from healthy donor muscle biopsies were cultured for less than XV passages and analyzed (**a**) in resting conditions and (**b**) after exposure to IFN-γ at 500 IU/ml for 48 hours. Expression of alkaline phosphatase (ALP) was assessed by ALP activity. The absence of CD56 expression, the quantification of CD44, CD146, CD13, CD49b pericyte markers and of CD117, CD45, CD31 lineage markers were evaluated by cytofluorimetric analysis. A representative sample from one out of eight MAB cells is shown.

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class I (median RFI: 20.5) but low levels of HLA class II (median RFI: 2.1), intracellular adhesion molecule-1 (ICAM-1; median RFI: 2.6), required to stabilize the immunological synapse,[21] and inhibitory costimulatory PDL-1 molecule (median RFI: 1.7). After exposure to IFN-γ, mesoangioblasts upregulate HLA class I (median RFI: 64.8; $P < 0.001$) (Figure 2a), HLA class II (median RFI: 16.0; $P < 0.001$) (Figure 2b), ICAM-1 (median RFI: 39.2; $P < 0.001$) (Figure 2c), and PDL-1 (median RFI: 4.5; $P < 0.05$) (Figure 2d). Activating costimulatory receptors CD80, CD86, CD40, and CD70 were not detectable on MAB nor on MABγ cell surface, while the basal expression of the LFA-3 adhesion molecule was unaltered upon IFN-γ exposure (median RFI: MAB 3.0; MABγ 2.9) (**Supplementary Table S1**). Upon exposure to TNF-α, another proinflammatory cytokine detectable in DMD muscles, expression of ICAM-1 increased significantly, while HLA-I, HLA-II, and PDL-1 were not upregulated (**Supplementary Figure S1**). IFN-γ treatment of MAB resulted in a broader upregulation of the molecules involved in the immunological synapse in comparison with TNF-α and was for that reason preferred to TNF-α for further analysis.

While T-cell receptor triggering and costimulatory molecule engagement are required for T-cell activation, polarization and acquisition of T-cell functions highly depend on cytokine-mediated signaling.[22] We then analyzed the cytokines secreted by MAB and observed a similar profile in resting and inflammatory conditions. Mature (mDC) and immature (iDC) dendritic cells were analyzed as controls. A reduction, although not significant, was observed in the secreted amount of the IL8 (average MAB: 2,528 pg/ml; MABγ: 1,138 pg/ml) and IL6 (average MAB: 625 pg/ml; MABγ: 859 pg/ml) myokines upon IFN-γ exposure of MAB. The tolerogenic IL10 and the proinflammatory TNF-α and IL12p70 cytokines were not expressed by MAB nor by MABγ (Figure 2e).

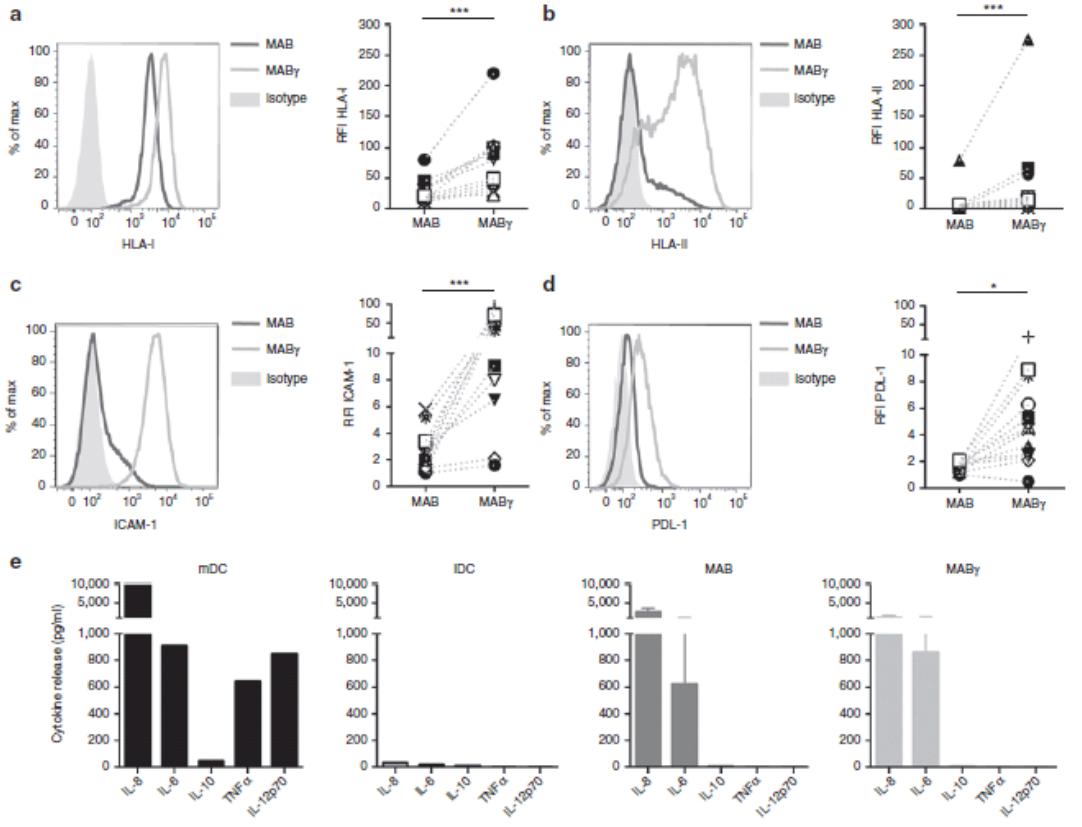

Figure 2 Human mesoangioblasts (MAB) acquire an immunologically active profile upon exposure to interferon-γ (IFN-γ). The levels of expression of (**a**) human leukocyte antigen (HLA) class I, (**b**) HLA class II, (**c**) intracellular adhesion molecule-1 (ICAM-1), and (**d**) PDL-1 were quantified by cytofluorimetric analysis. In the representative histograms, the dark line stands for resting MAB, the light line for MAB exposed to IFN-γ (MABγ), and the filled histograms for isotype controls (left panels). Relative fluorescence intensity (RFI) was measured as the ratio of the mean fluorescence intensity (MFI) of specific markers to the MFI of isotype controls. Each symbol represents the RFI of HLA class I, HLA class II, ICAM-1, and PDL-1 in individual donors, untreated, and IFN-γ treated. Cells harvested from individual donors are represented in all plots by the same symbol (right panels). (**e**) Cytokine secretion profile of MAB was determined by cytometric bead array. iDC, immature dendritic cells; MAB, mesoangioblasts in resting conditions; MABγ, mesoangioblasts after IFN-γ exposure; mDC, mature dendritic cells. $^*P < 0.05$; $^{***}P < 0.001$.

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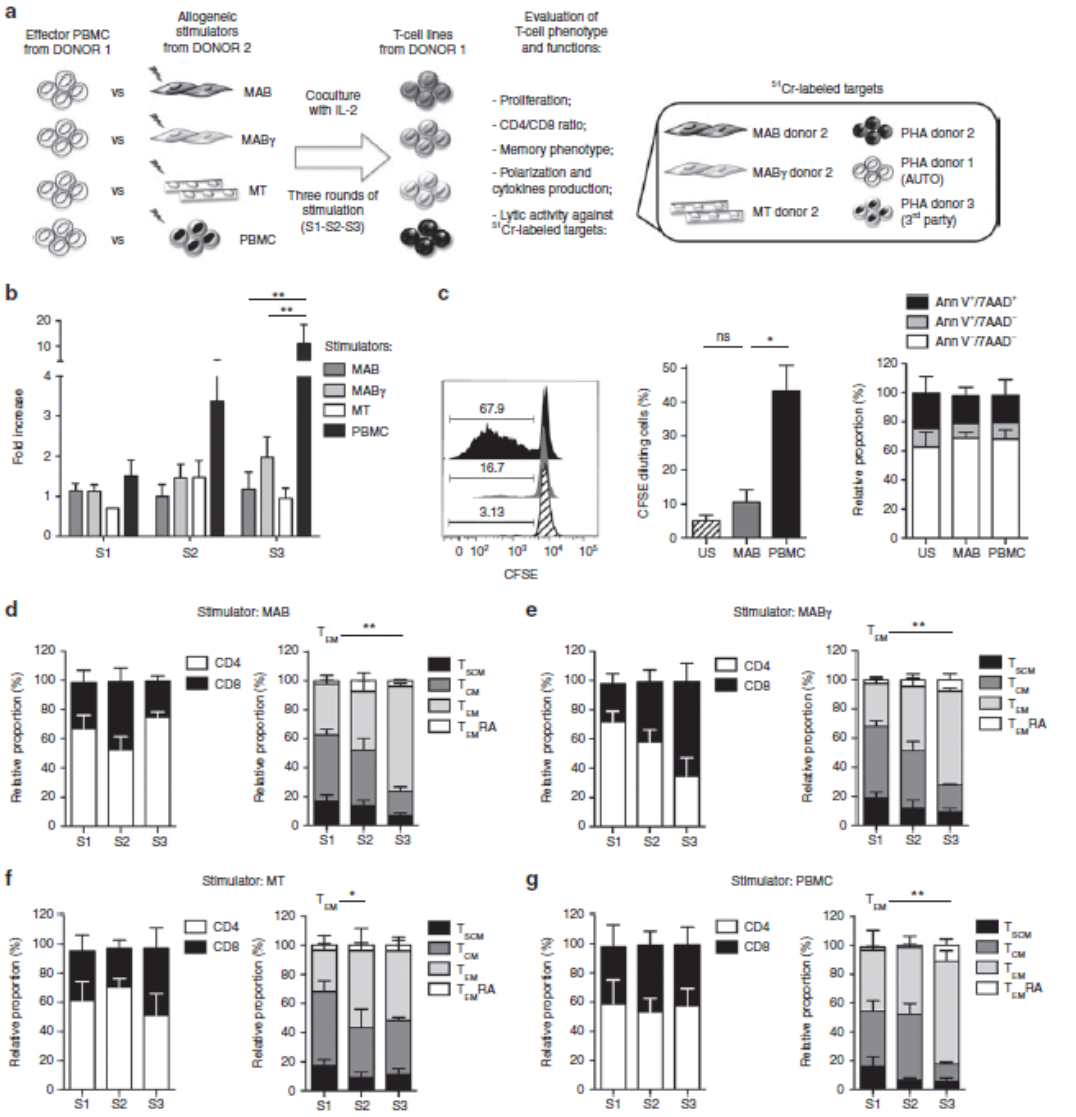
Overall, these analyses show that MAB display a basal hypoimmunogenic phenotypic profile that can be partially reverted in an inflammatory *milieu*.

MAB are less effective than peripheral blood mononuclear cells in promoting the expansion of allogeneic T cells

To test the ability of MAB in promoting alloreactive T-cell responses, peripheral blood mononuclear cells (PBMC) from healthy donors were cultured *in vitro* with irradiated allogeneic MAB, MABγ, and myotubes differentiated from MAB (MT). Irradiated PBMC were also harvested from MAB donors and used as positive controls. Cells were cultured in the presence of IL2 (60 IU/ml) and were restimulated and counted every 10–14 days. An outline of the experiments is shown in **Figure 3a**. Alloreactive T cells failed to expand upon stimulation with MAB and MT (average fold increase after three stimulations, S3: MAB: 1.2; MT: 1.0). IFN-γ pretreatment had limited effects (average fold increase at S3: 2), while allogeneic PBMC matched to MAB donor were significantly superior to MAB in promoting T-cell expansion (average fold increase at S3: 10.9; $P < 0.01$) (**Figure 3b**). We then stained effectors with carboxyfluorescein



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succinimidyl ester (CFSE) at the first day of culture and analyzed CFSE dilution, Annexin V expression, and 7AAD binding at day 7. As a negative control, the baseline proliferation of unstimulated PBMC kept in culture in the presence of IL2 at 60 UI/ml was measured (average CFSE diluting cells: 5.1%). T-cell proliferation upon MAB stimulation was significantly lower than that observed upon stimulation with PBMC (average CFSE diluting cells: 13.3% with MAB, 40.0% with PBMC, $P < 0.01$). The relative proportion of Annexin V/7AAD double negative T cells was similar in the two conditions, indicating that the different expansion rate promoted by MAB and PBMC was not due to a differential level of cell death but rather to a differential T-cell proliferation rate (Figure 3c). The phenotype of responder T cells was characterized after allogeneic stimulation with MAB (Figure 3d), MABγ (Figure 3e), MT (Figure 3f), and PBMC (Figure 3g). Throughout the culture, the CD4/CD8 ratio was maintained at physiological levels, except upon MABγ stimulation, which promoted a preferential $CD8^+$ expansion. Alloreactive T cells progressively acquired an effector memory ($CD62L^-/CD45RA^-$) phenotype independently from the nature of the stimulus.

MAB stimulate alloreactive T cells

To analyze the ability of MAB in shaping T-cell function, the polarization of stimulated T cells was evaluated (Figure 3a). We did not observe differences in the polarization (Figure 4, left panels) of alloreactive T cells expanded with MAB (Figure 4a), MABγ (Figure 4b), MT (Figure 4c) or PBMC (Figure 4d) harvested from the same donors. In particular, we did not observe expansion of naturally occurring regulatory T cells (T_{regs}) nor of IL10-producing cells in any of the conditions tested. Expanded T cells displayed a preferential T_{H1}/T_{C1} polarization, characterized by production of IFN-γ, in the absence of IL4. In order to evaluate the specificity and lytic activity of effectors expanded with different stimuli, a ^{51}Chromium release assay was performed against MAB, MABγ, MT, and a PHA T-cell line, from donor 2 (Figure 3a) at the end of the second stimulation (Figure 4, right panels). The effectors stimulated with MAB failed to recognize and kill MAB even at a E:T ratio of 50:1 (average lysis: 12.6%) but were lytic when challenged with other targets matched to MAB donor such as the PHA line (average lysis: 30.0%; $P < 0.001$), MABγ (average lysis: 26.4%; $P < 0.01$), and MT (average lysis: 22.8%; $P < 0.05$). Negative controls, a PHA T-cell line autologous to effector cells (AUTO, donor 1) and a PHA T-cell line harvested from a third donor (third party, donor 3), were not lysed (Figure 4a). Similarly, T cells stimulated with allogeneic MABγ (Figure 4b), MT (Figure 4c), and PBMC (Figure 4d) proved specifically cytotoxic against all matched targets, but again failed in lysing MAB. These results indicate that MAB are able to elicit an alloreactive T-cell response but are unexpectedly resistant to lysis by alloreactive T cells. Such resistance is reverted in inflammatory conditions and upon differentiation in myotubes.

Human MAB are resistant to T-cell killing in resting conditions but become sensitive upon IFN-γ treatment or differentiation in myotubes

To elucidate this phenomenon, the sensitivity of MAB to T-cell killing was further investigated. We initially exploited a T-cell clone specific for the HLA-A02–restricted HY_{FID} peptide, a naturally processed peptide derived from the HY minor histocompatibility antigen and encoded by the Y chromosome. MAB, MABγ, MT, and a lymphoblastoid cell line (LCL) were generated from $HLA\text{-}A02^+$ male donors and used as targets in a lytic assay. A maximum of 2.3% of MAB lysis was observed at an E:T ratio of 50:1, while MABγ (average lysis: 20.3%; $P < 0.01$) and MT (average lysis: 25.3%; $P < 0.01$) displayed a higher sensitivity to cytotoxic T-cells. The LCL line used as a positive control was killed significantly more than other targets (39.0% of lysis) (Figure 5a, left). Cells from $HLA\text{-}A02^-$ male donors, used as negative controls, were not lysed (Figure 5a, right). To verify whether resistance to T-cell killing displayed by MAB was due to a defective antigen processing or to the low level of peptide presentation, we pulsed $HLA\text{-}A02^+$ MAB with the HY_{FID}-specific peptide. No differences in sensitivity to T-cell killing were observed in MAB presenting the physiologically processed HY peptide and in HY_{FID}-pulsed MAB (average lysis: 3.4%), indicating that antigen processing is not the limiting step for MAB killing (Figure 5a, left). To confirm our results with a nominal antigen, a T clone specific for the HLA-A02–restricted cytomegalovirus (CMV) $pp65_{NLV}$ peptide was then used. MAB, MABγ, MT, and LCL lines isolated from $HLA\text{-}A02^+$ donors were pulsed with the $pp65_{NLV}$ peptide and tested as targets. An average of 17.8% of MAB lysis was reached at an E:T ratio of 50:1,

Figure 3 Mesoangioblasts (MAB) are less effective than peripheral blood mononuclear cells (PBMC) in promoting the expansion of allogeneic T cells. (**a**) Outline of the experiment. PBMC from healthy donors (donor 1) were cultured *in vitro* with irradiated allogeneic MAB, interferon-γ (IFN-γ)-treated MAB (MABγ), myotubes differentiated from MAB (MT), and PBMC harvested from the same donor (donor 2). Cells were cultured in the presence of IL2 (60 IU/ml) and were restimulated every 10–14 days. Phenotype and function of T-cell lines derived from donor 1 were characterized. After stimulation, T cells were challenged in ^{51}Chromium release assay against the same MAB, MABγ, MT, and a PHA T-cell line, from donor 2. Every effector cell was challenged with all targets and with negative controls represented by a PHA T-cell line autologous to effector cells (donor 1) and a PHA T-cell line harvested from a third donor (third party, donor 3). The experiment has been repeated seven times using different donors. (**b**) T-cell expansion (measured as fold increase) was analyzed for each culture. (**c**) Proliferation and cell death were measured by fluorescence-activated cell sorting (FACS) analysis in T cells stimulated with MAB and PBMC during the first round of stimulation. In the histogram of CFSE dilution measured 7 days after the beginning of culture, the proliferation of T cells stimulated with irradiated MAB (gray) and irradiated PBMC (black) and of unstimulated T-cells (dashed bar) are represented (left panel). Graphs depict average and SEM of the percentages of CFSE diluting cells (middle panel) and relative proportions of apoptotic cells, defined after Annexin V staining and 7AAD binding as nonapoptotic ($Annexin\ V^-/7AAD^-$), early apoptotic ($Annexin\ V^+/7AAD^-$) and late apoptotic ($Annexin\ V^+/7AAD^+$) (right panel). (**d**) The relative proportion of $CD4^+$ and $CD8^+$ subsets in responder T cells stimulated with allogeneic MAB was measured by FACS analysis 10–14 days after each round of stimulation (left panel). Quantification of differentiation stages in responder T cells was measured by FACS and defined after CD62L and CD45RA staining as memory stem T cells (T_{SCM}; $CD62L^+\ CD45RA^+$),[46] central memory (T_{CM}; $CD62L^+\ CD45RA^-$), effector memory ($T_{EM}CD62L^-\ CD45RA^-$), and terminal effectors ($T_{EM}Ra$; $CD62L^-\ CD45RA^+$) (right panel). Relative proportion of T-cell subsets and differentiation stages was quantified in T cells also after stimulation with (**e**) allogeneic MABγ, (**f**) MT, and (**g**) donor-matched PBMC. Averages and SEM are shown. $^*P < 0.05$; $^{**}P < 0.01$. CFSE, carboxyfluorescein succinimidyl ester.

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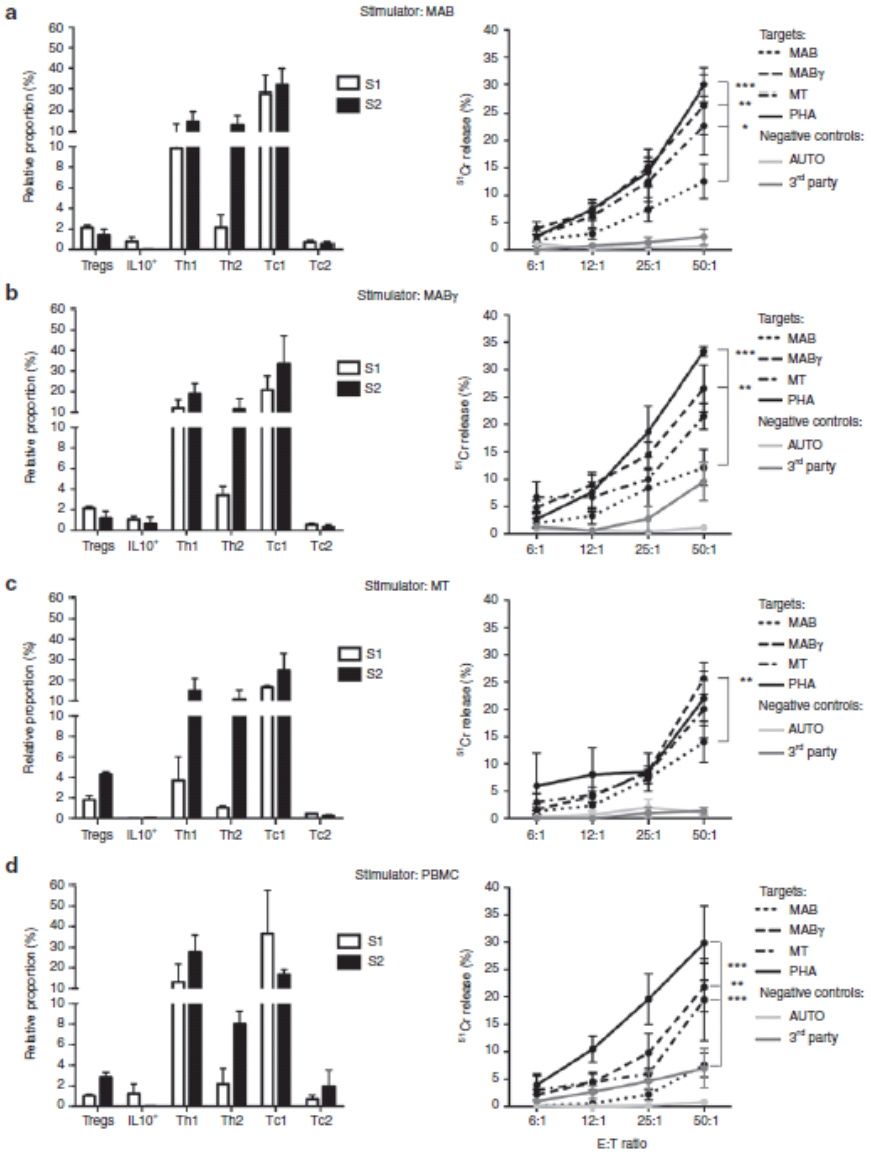

Figure 4 Mesoangioblasts (MAB) promote T-cell alloreactivity. T cells stimulated with (**a**) MAB, (**b**) MABγ, (**c**) MT, and (**d**) peripheral blood mononuclear cells (PBMC) were characterized and T-cell subsets were quantified, based on surface markers and cytokine secretion profiles. T lymphocytes were stained and analyzed by fluorescence-activated cell sorting 10–14 days after 1 (S1) and 2 (S2) rounds of stimulation. Natural regulatory T cells (T_{reg}) were defined as $CD4^+/CD25^{bright}/FoxP3^+/CD127^{low}$, $IL10^+$-secreting T cells ($IL10^+$) were defined as $CD4^+$ $IL10^+$ cells, T_{H1} as $CD4^+$ $IL4^-$ IFN-γ^+, T_{H2} as $CD4^+$ $IL4^+$ IFN-γ^-, T_{C1} as $CD8^+$ $IL4^-$ IFN-γ^+ and T_{C2} as $CD8^+$ $IL4^+$ IFN-γ^- (left panels). Lytic activity of responder T lymphocytes was measured 14 days after the second round of stimulation against allogeneic MAB and donor-matched MABγ, MT, and PHA T-cell line by ^{51}Chromium release assay. Autologous lymphocytes and third party targets were used as negative controls (right panels). Averages and SEM are shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

while MABγ (average lysis: 40.8%; $P < 0.05$), MT (average lysis: 43.8%; $P < 0.01$), and LCL (average lysis: 60.5%; $P < 0.001$) again displayed a higher sensitivity to T-cell killing (Figure 5b, left). Cells from HLA-A02$^+$ donors pulsed with an irrelevant peptide were used as negative controls and were not lysed (Figure 5b, right). Thus, MAB are intrinsically resistant to T-cell killing, and inflammatory signals might revert this resistance. The differential sensitivity to T-cell killing displayed by MAB, MABγ and MT was not completely explained

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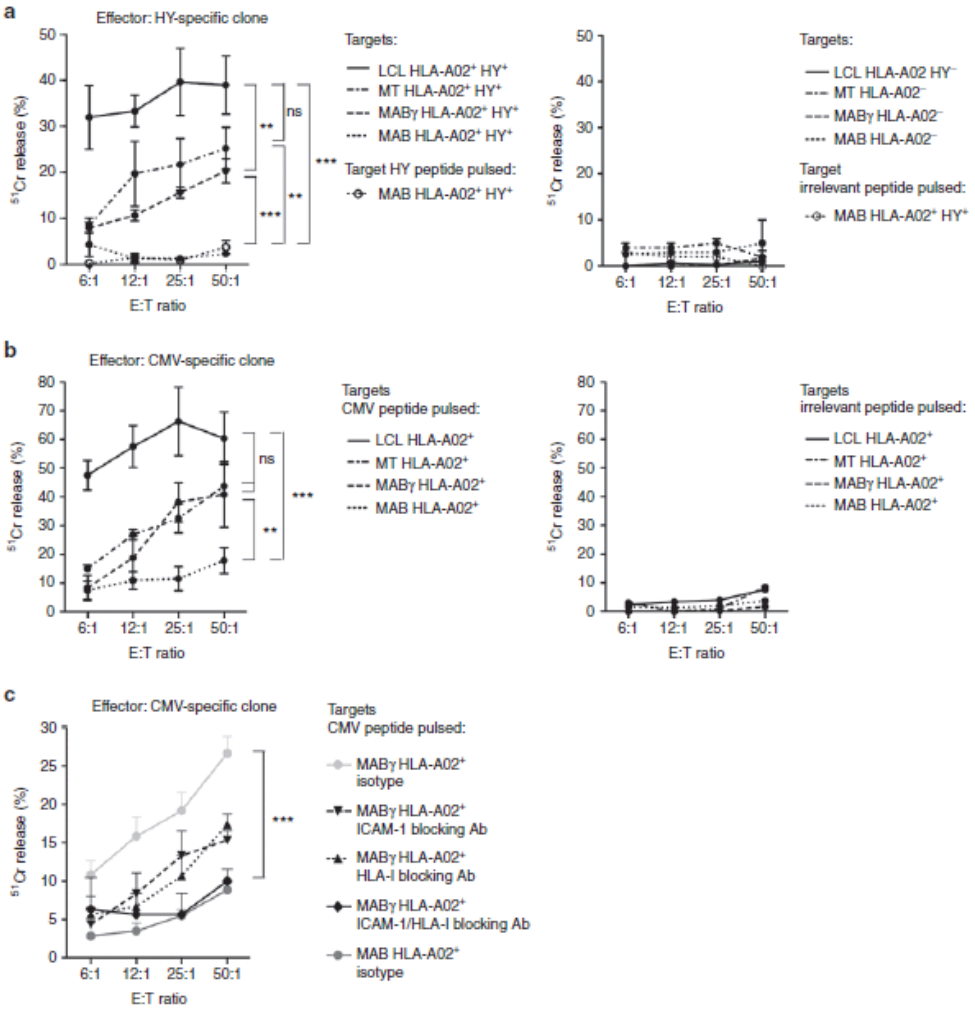

Figure 5 Human mesoangioblasts (MAB) are resistant to T-cell killing. T-cell clones were tested with specific targets: MAB, MABγ, MT, and lymphoblastoid cell line (LCL) lines as positive control. (**a**) Lytic activity of a T-cell clone specific for the minor histocompatibility antigen HY_{EID}-restricted by human leukocyte antigen (HLA)-A02 is shown. MAB, MABγ, MT, and LCL lines isolated from HLA-A02+ male donors were used as targets. MAB pulsed with the HY_{EID}-specific peptide were also tested (left panel). MAB, MABγ, MT, and LCL lines isolated from HLA-A02- donors were used as negative controls (right panel). (**b**) Lytic activity of a T clone specific for the cytomegalovirus (CMV) $pp65_{NLV}$ peptide restricted by HLA-A02 is shown. MAB, MABγ, MT, and LCL lines isolated from HLA-A02+ donors and pulsed with the $pp65_{NLV}$-specific peptide were tested as targets (left panel). MAB, MABγ, MT, and LCL lines isolated from HLA-A02+ donors and pulsed with an irrelevant peptide were tested as negative controls (right panel). (**c**) Lytic activity of the $pp65_{NLV}$/HLA-A2–restricted T clone was tested against $pp65_{NLV}$-pulsed MABγ in the presence of the intracellular adhesion molecule-1 (ICAM-1)-blocking antibody, HLA-ABC–blocking antibody, a combination of the two antibodies, or of the isotype control. Averages and SEM are shown. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

by their different levels of HLA class I expression, since MT and MAB showed a different sensitivity to T-cell killing, despite similar levels of HLA class I expression (**Supplementary Figure S2**). In blocking experiments, we observed that, although both ICAM-1 and HLA-I blocking were effective in reducing sensitivity to T-cell killing, the combined neutralization was required to reduce the sensitivity to lysis of MABγ to that observed with MAB (Figure 5c).

Protease Inhibitor-9 downmodulation by shRNA enhances MAB susceptibility to T-cell killing

Since T-cell killing is largely mediated by the FAS/FASL interaction, we verified FAS expression on MAB and we observed that FAS is homogeneously expressed on MAB at high levels (average RFI: MAB 10.1; MABγ 8.9) similar to levels measured on LCL (average RFI: 10.5) (Figure 6a). Nevertheless, the addition of a FAS agonist induced a lower level of apoptosis in MAB

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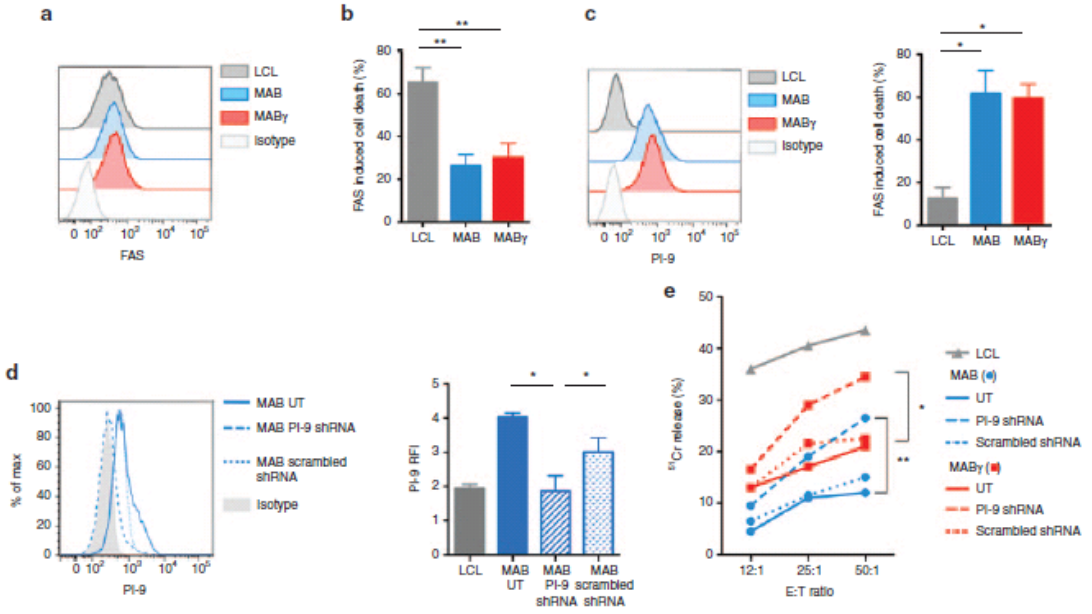

Figure 6 Protease inhibitor-9 (PI-9) downmodulation by shRNA enhances mesoangioblasts (MAB) sensitivity to T-cell killing. (**a**) FAS expression on MAB and lymphoblastoid cell line (LCL) lines was quantified by fluorescence-activated cell sorting (FACS) analysis. In the histograms, the level of expression of FAS on LCL (gray), MAB (blue), MABγ (red), and isotype control (light) is represented. (**b**) FAS signaling was tested by exposure of MAB, MABγ, and LCL lines to CH11 FAS-specific activating antibody (15 µg/ml). Cell death was measured by Annexin V staining and 7AAD binding flow cytometric assay. Results are shown as the difference between the relative proportion of Annexin V$^+$ and/or 7AAD$^+$ cells in FAS-triggered samples and in cells exposed to isotype control. (**c**) The expression of the serine protease inhibitor PI-9 on MAB and LCL lines was quantified by FACS analysis. In the histograms, the level of expression of PI-9 in LCL (gray), MAB (blue), MABγ (red), and isotype control (light) is represented (left panel). Average percentages of expressing cells are reported (right panel). (**d**) Downmodulation of PI-9 following specific shRNA transduction of MAB was evaluated by FACS analysis. In the histogram, the continuous line represents untransduced MAB (MAB UT), the dashed line represents MAB transduced with PI-9–specific shRNA (MAB PI-9 shRNA), the dotted line represents MAB transduced with scrambled RNA (MAB scrambled shRNA), and the filled histogram represents the isotype control (left panel). Average relative fluorescence intensity (RFI) of expressing cells are reported (right panel). (**e**) Lytic activity of a T clone specific for the cytomegalovirus (CMV) pp65$_{NLV}$ peptide restricted by human leukocyte antigen (HLA)-A02 was measured by ^{51}Chromium release assay. MAB (blue) and MABγ (red) isolated from HLA-A02$^+$ donors were kept untransduced (continuous lines), transduced with PI-9–specific shRNA (dashed lines), and transduced with nonspecific scrambled shRNA (dotted lines). An HLA-A02$^+$ LCL line was used as positive control (gray line). All targets were pulsed with the pp65$_{NLV}$-specific peptide or an irrelevant peptide. Results are expressed as the difference between % of lysis calculated with CMV-specific peptide and that observed with the control peptide. *$P < 0.05$; **$P < 0.01$.

(average cell death: 26.1%) and MABγ (average cell death: 30.2%) than in LCL (average cell death: 65.3%; $P < 0.01$), suggesting that antiapoptotic mechanisms are active in these FAS$^+$ stem cells (Figure 6b). The serine protease inhibitor-9 (PI-9) is a potent inhibitor of apoptosis, we thus hypothesized that PI-9 expression could be involved in MAB resistance to T-cell killing. FACS analysis revealed a constitutive expression of intracellular PI-9 in MAB (average: 61.7%) and MABγ (average: 59.6%) as opposed to the low level of expression on LCL (average: 12.6%; $P < 0.05$) (Figure 6c). To verify the functional role of PI-9 in MAB resistance to T-cell killing, we transferred a shRNA specific for PI-9 in MAB by lentiviral vectors (LV). MAB were highly sensitive to LV-mediated transduction (mean transduction efficiency = 47.7% at multiplicity of infection: 2.5). In transduced MAB, the expression of PI-9 was significantly lower than in untreated MAB and MAB transduced with a scrambled shRNA (Figure 6d). We then exploited the T clone specific for the CMV pp65$_{NLV}$ peptide to verify the sensitivity of PI-9^{low} MAB to T-cell killing. MAB and MABγ isolated from HLA-A02$^+$ donors were transduced to express a PI-9 shRNA, a scrambled RNA or mock-transduced and used as targets. LCLs from HLA-A02$^+$ donors were used as positive controls. T-cell killing of PI-9^{low} MAB was 2-fold higher than killing of control MAB. A higher percentage of killing was observed also after PI-9 downmodulation in MABγ (27% average lysis at E:T ratio 50:1) if compared to that measured on untransduced MABγ (17% average lysis at E:T ratio 50:1; $P < 0.05$). The scrambled shRNA used as negative control did not affect MAB nor MABγ sensitivity to T-cell killing (Figure 6e). HLA-I levels were measured on GFP$^+$ cells and were similar between cells transduced with PI-9–specific shRNA (average RFI: 33.7) and cells transduced with the scrambled shRNA (average RFI: 31.5; data not shown). Thus, PI-9 expression affects MAB resistance to T-cell killing, independently from the level of expression of HLA-I, and represents a major mechanism of MAB immune evasion.

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DISCUSSION

Thanks to their ability to self-renew and differentiate into several cell types, stem cells represent promising therapeutic cellular products in regenerative medicine. In the past few years, several types of mesoderm-derived stem/progenitor cells were isolated from mice and humans and evaluated for their ability to differentiate into myofibers, in the attempt to treat muscular dystrophies.[23] Human MAB are pericyte-derived stem cells that can be isolated from adult skeletal muscle. At variance with other stem cells, such as mesenchymal stromal cells (MSCs), postnatal MAB have the ability to differentiate into skeletal muscle *in vitro* and *in vivo*. Additionally, the ability to grow extensively *in vitro* and to cross the endothelium makes the systemic delivery of MAB a promising therapeutic tool for muscle regeneration.[14,16,24] A phase 1–2 clinical trial testing the safety of intra-arterial infusion of allogeneic MAB in patients affected by DMD is currently ongoing in our Institute (EudraCT 2011-000176-33). For an efficient and persistent therapeutic effect, cellular products are required to evade immune responses that might lead to rejection of stem cells and/or their progeny. Several experimental evidences suggest that stem cells might possess immune privileged and immune modulatory properties. In human MSCs, defects in antigen presentation capacity and resistance to apoptosis have been described and associated to their ability to elude immune recognition.[25,26] In addition, MSCs inhibit T-cells proliferation by releasing soluble immunosuppressive factors and favor the generation of $CD4^+/CD25^+$ T regulatory cells.[27–29] Such immunosuppressive properties were exploited in hematopoietic stem cell transplantation to treat and prevent graft versus host disease,[30,31] autoimmunity, sepsis, and inflammatory diseases.[32,33] Nevertheless, a controlled randomized clinical trial failed in confirming the efficacy of MSCs in controlling graft versus host disease, leaving the discussion still controversial.[34] Interestingly, the allogeneic transplantation of mouse MAB into alpha-sarcoglycan null dystrophic mice, in the presence of immunosuppressive treatment, was followed by poor immune responses and resulted in the formation of alpha-sarcoglycan positive muscle fibers.[35] With the aim of evaluating the immunogenicity of human MAB and to predict their sensitivity to allogeneic rejection mechanisms when infused to DMD patients, we analyzed the immunological profile of MAB in resting conditions and after treatment with IFN-γ, to mimic the inflammatory *milieu* associated to dystrophic muscles.[7] We observed that inflammation partially reverts the hypoimmunogenic phenotypic profile of resting MAB. To challenge the phenotypic profile of MAB in functional assays, we compared MAB and donor-matched PBMC for the ability to elicit an alloreactive immune response. We observed that MAB are significantly less efficient than donor-matched PBMC in promoting the expansion of alloreactive lymphocytes. Of notice, IFN-γ treatment and differentiation in myotubes failed in rescuing this MAB immunological defect. These observations are in line with the suppressive activity of MAB, recently reported by English *et al.*[36,37] Interestingly, we observed that the few MAB-expanded alloreactive T cells display a preferential T_{H1}/T_{C1} cytokine secretion profile and do not express markers of regulatory T cells. Most importantly, MAB-expanded alloreactive T cells recognized and killed efficiently matched IFN-γ–treated MAB, myotubes, and matched T cells, thus showing a preserved cytotoxic effector function. Strikingly, resting MAB displayed a unique resistance to T-cell killing. This intriguing property was not due to a defect in antigen processing, since it was not rescued by peptide pulsing. The different levels of expression of HLA class I in target cells could not completely explain their differential sensitivity to T-cell killing, since MAB and MT showed similar levels of HLA class I expression but were differentially sensitive to lysis. Since antigen-specific T-cell clones recognized and killed efficiently IFN-γ–treated MAB, while sparing resting MAB, we initially investigated a possible role of ICAM-1, a molecule required for an effective immunological synapse[38] and expressed by MAB only after IFN-γ exposure. Only the combined neutralization of ICAM-1 and HLA-I restored on MABγ the resistance to apoptosis displayed by resting MAB, suggesting that even low levels of HLA class I expression permit MABγ killing when the immunological synapse is stabilized through engagement of adhesion molecules. We identified the serine PI-9, belonging to the serpin superfamily, as a major mechanism involved in MAB resistance to T-cell killing. PI-9 is a potent inhibitor of cell-induced apoptosis,[39,40] whose expression has been described as a mechanism of immune escape in MSCs[26] and immunoprivileged tissues.[41] We show here that PI-9 downmodulation in MAB by specific PI-9 shRNA enhances MAB sensitivity to T-cell killing by 2-folds. IFN-γ treatment did not affect PI-9 expression in MABγ, while resulting in higher susceptibility to T-cell killing. The results of neutralization experiments strongly indicate that the upregulation of HLA-I and ICAM-1 is crucial for an effective T-cell killing of MABγ and counteract this antiapoptotic mechanism. Besides the inhibition of Granzyme B and caspases exerted by serpins, prosurvival strategies may rely on the expression of Bcl-2 family members[42] or on the modulation of signal transduction of FAS[43] or other receptors involved in ligand-induced cell death. We might suppose that PI-9 expression synergise with antiapoptotic molecules to prevent MAB immune-mediated killing.

Overall, these results show that MAB have peculiar immunological privileges. Indeed, the few alloreactive T cells expanded upon MAB stimulation are fully immune competent and able to eliminate differentiated cells. Although a variable number of T cells might be expanded, depending on the quality of the stimulus, T cells stimulated with MAB or PBMC will be equally functional. The mechanism of stem cell immune evasion here described, therefore, does not rely on anergy and does not interfere with the development of a T cell-based immune response, but rather relies on an intrinsic resistance of stem cells to T-cell killing. Such a mechanism couples the development of functional immune responses with a selective preservation of self-renewing stem cell compartments. Inflammation and differentiation result in a MAB progeny sensitive to alloreactive T-cell killing, thus justifying and recommending the use of immunosuppressive and/or anti-inflammatory drugs in clinical trials exploiting the regenerative capacity of allogeneic MAB.

MATERIALS AND METHODS

Primary cells. MAB and PBMC were isolated from patients undergoing muscular biopsy and classified as nondystrophic, according to a protocol approved by the San Raffaele Ethical Committee. All donors gave their written informed consent to participate in agreement with the Declaration of Helsinki. MAB were maintained in culture in a medium consisted of MegaCell Dulbecco's modified Eagle medium (Sigma, St Louis, MO) supplemented with penicillin (100 UI/ml; Pharmacia, New York, NY),

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streptomycin (100 UI/ml; Bristol-Meyers Squibb, Rome, Italy), basic fibroblast growth factor (5 ng/ml; Peprotech, Rocky Hill, NJ), glutamine (2 mmol/l; Lonza, Basel, Switzerland), β-mercaptoethanol (0.1 mmol/l; GIBCO-BRL, Grand Island, NY), nonessential amino acids (1%; Sigma), and 5% fetal bovine serum (BioWhittaker-Lonza, Basel, Switzerland) as previously described.[20] Differentiation of MAB in myotubes (MT) was induced by plating cells onto Matrigel (BD Biosciences, San Jose, CA)-coated dishes in high-glucose Dulbecco's modified Eagle medium supplemented with 2% horse serum (Euroclone, Pero, Italy), glutamine (2 mmol/l), penicillin (100 UI/ml), and streptomycin (100 UI/ml) (differentiation medium).[20] PBMC were isolated by Ficoll-Hypaque gradient separation (Lymphoprep; Fresenius, Bad Homburg, Germany). Cells were cultured in Iscove's Modified Dulbecco's Media (GIBCO-BRL), supplemented with penicillin (100 UI/ml), streptomycin (100 UI/ml), and 10% fetal bovine serum (Iscove's Modified Dulbecco's Media complete medium).

MAB characterization. Mesoangioblasts were characterized in resting conditions and after exposure to IFN-γ (500 IU/ml; Peprotech) (MABγ) for 48 hours. The expression of lineage markers was analyzed by flow cytometry after co staining with anti-CD56-PE, anti-CD44-FITC, anti-CD146-PE, anti-CD13-PE, anti-CD49b-FITC, anti-CD117-FITC, anti-CD45-PE, and CD31-FITC antibodies (BD Biosciences). The alkaline phosphatase expression was assessed by alkaline phosphatase activity. The immunological characterization was performed by flow cytometry after staining with anti-HLA I-FITC, anti-HLA II-PE, anti-ICAM 1-PE, anti-CD86-FITC, anti CD80-PE, anti-CD40-FITC, anti-CD70-PE, anti-PDL-1-PE, and anti-LFA3-FITC antibodies. For the evaluation of cytokines released by MAB and MABγ, supernatant was collected 48 hours after plating cells in six-well plates and concentrations of IL8, IL1β, IL6, IL10, TNF-α, and IL12p70 were simultaneously measured using a Cytometric Beads Array Human Inflammatory Cytokines Kit (BD Biosciences) according to manufacturer instructions. FAS expression was measured by staining with anti-FAS-PE antibody (BD Biosciences) and intracytoplasmic staining of PI-9 was performed with anti-PI-9 antibody (Abcam, Cambridge, UK). Samples were analyzed on a FACS Canto I (BD Biosciences) with the FlowJo software (TreeStar, Ashland, OR).

Expansion of alloreactive T cells and T-cell clones. PBMC from healthy donors were cultured *in vitro* with allogeneic PBMC (irradiated at 30 Gy), MAB, MABγ, or MT (irradiated at 100 Gy). The different steric and biologic (adherent versus nonadherent) properties on MAB, MT, and PBMC did not allow to use exactly the same number of stimulators in all conditions. After preliminary experiments, we selected the conditions that preserved at best cell viability, *i.e.*, MAB, MABγ and MT semiconfluent, and PBMC (stimulators and effectors) 1×10^6/ml, leading to at a responder/stimulator ratio of 1/1 (PBMC) and 40/1 (MAB, MABγ, MT). Cells were kept in culture in Iscove's Modified Dulbecco's Media supplemented with glutamine (1%), penicillin (1%), streptomycin (1%), fetal bovine serum (10%), and IL2 at 60 UI/ml. IL2 was replaced every 3–4 days and responders were restimulated every 2 weeks. The medium used in the assay were the same for all conditions. Cell proliferation was determined by CFSE dilution, responders were labeled with CFSE (5 μmol/l) for 8 minutes at 37 °C and then stimulated with allogeneic targets for 7 days. We determined percentages of dividing cells, and percentages of dying cells, upon staining with anti-Annexin V-PB and 7AAD binding, and data acquisition on a FACS Canto I (BD Biosciences) with the FlowJo software (TreeStar). CMV-specific T-cell lines and clones were generated from PBMC harvested from an HLA-A02$^+$ CMV seropositive healthy donor. Cells were expanded by rapid expansion protocol.[44] Briefly, 10^5–10^6 cells were cultured in the presence of 25×10^6 allogeneic PBMC (irradiated at 30 Gy) and 5×10^6 allogeneic EBV LCL (irradiated at 100 Gy) in medium containing OKT3 (30 ng/ml) and IL2 (600 UI/ml). HY-specific T-cell clones were kindly provided from Els Goulmy, Department of Immunohematology and Blood Bank, Leiden University Medical Center. Cells were expanded in the presence of: 20×10^6 allogeneic PBMC (irradiated at 30 Gy) and 4×10^6 allogeneic LCL isolated from HLA-A02$^+$ male donors (irradiated at 100 Gy) in medium containing leucoagglutinin A (1.5%) and IL2 (200 UI/ml).

Functional assays on alloreactive T cells and specific T-cell clones. T cells cultured with allogeneic targets were analyzed after each stimulation by flow cytometry after staining with anti-CD3-FITC, anti-CD62L-PE, anti-CD8-PerCP, anti-CD4-APC H7, anti-CD45RA-PB, and anti-CD56-PE-Cy7 antibodies (BD Biosciences; Biolegend, San Diego, CA). T_{regs} frequencies were determined by flow cytometry as percentages of CD4$^+$ cells displaying a CD127^{low} Foxp3$^+$ CD25^{bright} phenotype. The relative proportion of T_{H1}/T_{C1}-, T_{H2}/T_{C2}-, and IL10-producing cells was determined by flow cytometry after intracellular staining with anti-IFN-γ-FITC, anti-IL4-PE, or anti-IL10-PE antibodies on T cells activated for 6 hours with phorbol 12-myristate 13-acetate (50 ng/ml)/ionomycin (1 μg/ml). Cytotoxic activity was measured by a standard ^{51}Cr release assay at increasing effector/target (E/T) ratios. Alloreactive T cells and T-cell clones were incubated for 4 hours with ^{51}Cr-labeled targets. Specific lysis was expressed according to the following formula: 100 × (average experimental cpm – average spontaneous cpm)/(average maximum cpm – average spontaneous cpm). In peptide pulsing experiments, targets were incubated for 1 hour at 37 °C with the synthetic HY_{FID} (10 μg/ml) or CMV $pp65_{NLV}$ peptide (10 μg/ml). In blocking experiments, target cells were incubated for 1 hour at room temperature with a neutralizing anti-ICAM-1 antibody (10 μg/ml, clone BBIG-I1; R&D System, Minneapolis, MN) and with a neutralizing anti-HLA-ABC antibody (30 μg/ml, clone W6/32; Biolegend).

Immunofluorescence. Cells were fixed with 4% paraformaldehyde for 10 minutes at 4 °C. After washes, blocking and permeabilization were performed in phosphate-buffered saline–Triton 0.1% supplemented with 10% normal goat serum (Jackson, West Grove, PA) for 45 minutes at room temperature. Cells were then incubated for 1 hour at room temperature with the MF20 IgG_{2b} antibody (Developmental Hybridoma Bank, Iowa City, IA) that recognizes all sarcomeric myosin heavy chains, diluted 1/5, and the mouse anti-HLA-ABC IgG_{2a} antibody clone W6/32 (Biolegend), diluted 1/50. Cells were then washed and incubated for 1 hour at room temperature in the dark with secondary antibodies diluted 1/500 (goat anti-mouse IgG_{2a} Alexa Fluor 546 and goat anti-mouse IgG_{2b} Alexa Fluor 488; Invitrogen, Carlsbad, CA). Hoechst was applied 1:1,000 to stain nuclei. Images were acquired with an Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany) and quantitative analyses were performed with ImageJ software.

Apoptosis induction. For induction of apoptosis, cells were treated with 15 μg/ml of the agonistic anti-FAS antibody (clone CH11; Millipore, Billerica, MA). After 24 hours, apoptosis was measured by staining with anti-Annexin V-FITC and by 7AAD binding (BD Biosciences).

Short hairpin RNA knockdown of PI-9 in MAB. Small hairpin RNA (shRNA) against PI-9 was purchased from Dharmacon (GIPZ lentiviral shRNA target gene set and nonsilencing-GIPZ lentiviral shRNA control; Thermo Fisher Scientific-Dharmacon Products, Lafayette, CO). MAB were transduced as previously reported.[45] Briefly, MAB were plated at 100,000/well in six-well plates. PI-9 and nonspecific scrambled shRNA (multiplicity of infection: 2.5) were added to MAB. After 5 days incubation, MAB were harvested, transduction efficiency was assessed by quantifying GFP expressing cells and PI-9 expression was quantified by flow cytometry. Transduced MAB were FACS-purified on a MoFlo MLS cell sorter (DAKO, Glostrup, Denmark) and were subjected to the killing assay as described above. Post-sorting analysis of purified GFP$^+$ cells revealed >95% purity.

Statistical analysis. One-way analysis of variance with Bonferroni correction and the two-tailed paired *t*-test were used for comparison of three

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or more groups, or two groups, respectively. Statistical analyses were performed with Prism 5 (GraphPad Software).

SUPPLEMENTARY MATERIAL

Figure S1. IFN-γ and TNF-α treatment of MAB results in upregulation of molecules involved in the immunological synapse.
Figure S2. Levels of expression of HLA class I on target cells.
Table S1. Immunological characterization of untreated, IFN-γ treated and TNF-α treated mesoangioblasts.

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Intra-Arterial Transplantation of HLA-Matched Donor Mesoangioblasts in Duchenne Muscular Dystrophy

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Abstract:	BACKGROUND Intra-arterial transplantation of mesoangioblast progenitors proved safe and partially efficacious in pre-clinical models of muscular dystrophy. After completion of a toxicity study and an observational study on 28 DMD patients, five (age 8.5 to 12.4, two wheelchair-bound) were selected for a first-in-human, exploratory, prospective, sequential, non-randomized open-label phase I-IIa clinical trial of intra-arterial HLA-matched donor cell transplantation. METHODS We administered escalating doses (3.3, 6.6, and 19.8 x106 cells/Kg) of donor-derived mesoangioblasts in limb arteries under immune-suppressive therapy (tacrolimus). Four consecutive infusions were performed at two months intervals, preceded and followed by clinical and laboratory tests. Muscular MRI was performed after the first infusion and then every 6 months. Two months after the last infusion a muscle biopsy was analyzed for the presence of donor derived DNA and dystrophin transcript and protein. Safety was the primary endpoint. RESULTS The study was safe with an asymptomatic thalamic stroke detected in one patient. The cause of stroke and correlation with mesoangioblast infusion remained unclear. No alterations of cardiac or respiratory functions were detected. MRI documented the advanced phase progression of the disease in 4 patients; in one patient, minimal disease progression was observed. Functional measures were transiently stabilized in 2 of 3 ambulant patients. Donor DNA was detected in muscle biopsies of 4 out of 5 patients and donor derived dystrophin in one. CONCLUSIONS Intra-arterial transplantation of donor mesoangioblasts in human proved to be feasible and safe. Although our study was not designed to measure efficacy, results support future studies.

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Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne Muscular Dystrophy.

Giulio Cossu, M.D., Stefano C. Previtali, M.D. Ph.D, Sara Napolitano MD, Maria Pia Cicalese, M.D., Francesco Saverio Tedesco, M.D. PhD, Francesca Nicastro, P.T., Maddalena Noviello, Ph.D., Urmas Roostalu, Ph.D., Maria Grazia Natali Sora, M.D., Marina Scarlato, M.D., PhD, Maurizio De Pellegrin, M.D., Claudia Godi, M.D., Serena Giuliani, M.S.W., Francesca Ciotti, M.S.W, Rossana Tonlorenzi, Ph.D., Isabella Lorenzetti, M.Sc., Cristina Rivellini, M.Sc., Sara Benedetti, Ph.D., Roberto Gatti, P.T., M.Sc., Sarah Marktel, M.D., Benedetta Mazzi, M.Sc, Andrea Tettamanti, P.T., M.Sc., Martina Ragazzi, M.Sc, Maria Adele Imro, Ph.D., Giuseppina Marano, M.Sc., Rossana Fiori, M.D., Maria Pia Sormani, Ph.D., Chiara Bonini, M.D., Letterio S. Politi, M.D., Massimo Venturini, M.D., Yvan Torrente, M.D. Ph.D and Fabio Ciceri, M.D.

From the Institute of Inflammation and Repair, University of Manchester UK (G.C., U.R.); Institute of Experimental Neurology (Inspe) and Division of Neuroscience (S.C.P., R.T., I.L., C.R.), Department of Neurology (S.C.P, M.G.N.S, M.S.), HSRTIGET Pediatric Clinical Research Unit (MPC, SN, FC), Hematology and BMT Unit (S.N., M.P.C., S.G., S.M., F.C.), Immunogenetics Laboratory, Dept. of Immunoematology & Blood Transfusion (B.M.) Experimental Hematology Unit, Division of Immunology, Transplantation and Infectious Diseases (M.N., C.B.), Neuroradiology Department and Neuroradiology Research Unit (C.G., L.S.P.), Unit of Orthopaedics (M.DP), Unit of Anesthesiology (R.F.), Department of Radiology (M.V.), Laboratory of analysis and rehabilitation of motor function, Division of Neurosciences, (F.N., A.T., R.G.), IRCCS San Raffaele Scientific Institute, Milan, Italy; MolMed S.p.A., Milan, Italy (M.A.I., G.M.), Department of Cell and Developmental Biology, University College London, UK (F.S.T., S.B, M.R.); Department of Health Sciences, University of Genoa (M.P.S.) Italy; Stem Cell Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Italy (Y.T., C.G., F.N.)

Correspondence to GC (giulio.cossu@manchester.ac.uk), SCP (previtali.stefano@hsr.it), YT (yvan.torrente@unimi.it) or FC (ciceri.fabio@hsr.it)

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ABSTRACT

BACKGROUND

Intra-arterial transplantation of mesoangioblast progenitors proved safe and partially efficacious in pre-clinical models of muscular dystrophy. After completion of a toxicity study and an observational study on 28 DMD patients, five (age 8.5 to 12.4, two wheelchair-bound) were selected for a first-in-human, exploratory, prospective, sequential, non-randomized open-label phase I-IIa clinical trial of intra-arterial HLA-matched donor cell transplantation.

METHODS

We administered escalating doses (3.3, 6.6, and 19.8 $x10^6$ cells/Kg) of donor-derived mesoangioblasts in limb arteries under immune-suppressive therapy (tacrolimus). Four consecutive infusions were performed at two months intervals, preceded and followed by clinical and laboratory tests. Muscular MRI was performed after the first infusion and then every 6 months. Two months after the last infusion a muscle biopsy was analyzed for the presence of donor derived DNA and dystrophin transcript and protein. Safety was the primary endpoint.

RESULTS

The study was safe with an asymptomatic thalamic stroke detected in one patient. The cause of stroke and correlation with mesoangioblast infusion remained unclear. No alterations of cardiac or respiratory functions were detected. MRI documented the advanced phase progression of the disease in 4 patients; in one patient, minimal disease progression was observed. Functional measures were transiently stabilized in 2 of 3 ambulant patients. Donor DNA was detected in muscle biopsies of 4 out of 5 patients and donor derived dystrophin in one.

CONCLUSIONS

Intra-arterial transplantation of donor mesoangioblasts in human proved to be feasible and safe. Although our study was not designed to measure efficacy, results support future studies.

Duchenne Muscular Dystrophy (DMD) is the most common muscular dystrophy, due to mutations of the X-linked dystrophin gene.[1,2] It causes a progressive degeneration of skeletal and cardiac muscle, leading the patient to reduced motility, wheelchair confinement and early death, usually due to cardiac and/or respiratory failure.[3,4] Drug and physical therapy have extended patients' lifespan but only modestly its quality[5,6].

A number of new therapies for DMD have entered clinical development and some have progressed to phase III.[7-11] All appear to be safe but are often limited to a subset of patients and long lasting efficacy has still to be reached. In the past, we have characterized mesoangioblasts (MABs), a subset of pericytes, from mouse, dog and human skeletal muscle that can be expanded in culture and maintain the ability to differentiate into skeletal and smooth muscle.[12-14] Noteworthy, MABs are able to cross the vessel wall when delivered intra-arterially and can thus being distributed to downstream tissues, provided that inflammation and consequent activation of the endothelium are present, as in disease progression of DMD. We tested a protocol of cell therapy in three murine and one canine model of muscular dystrophies, demonstrating safety and partial efficacy of this approach.[15-19]

Following an observational study in 28 DMD patients aimed at defining longitudinally the disease progression,[20] five DMD patients were enrolled to a "first-in-human" phase I/IIa trial of HLA-matched sibling donor MABs under immune suppression.

METHODS

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Detailed Methods are presented in supplemental appendix.

STUDY DESIGN, MEDICINAL PRODUCT AND CELL ENGRAFTMENT

Five patients (details in Table 1) underwent transplantation of MABs obtained from a muscle biopsy of an HLA-matched brother. Target dose of MABs was consistent to doses administered to dystrophic dogs in pre-clinical tests.[16] All patients received at least four infusions (Suppl. Fig. 1 and appendix) in upper and lower (Pt01, 02, 03) or only lower limbs (Pt05. 06), under immunosuppression regimen (tacrolimus).

MolMed (http://www.molmed.com) produced and released human donor MABs as Medicinal Product (MP). Good Manufacturing Practices (GMP) were applied to scale up the process of isolation, characterization and expansion.[21] The MP showed myogenic potency as cells spontaneously differentiated into myotubes *in vitro* (Suppl. Fig. 2). MP was infused (concentration of $5x10^6$/ml in normal saline solution) at a rate of 1 or 2 ml/min by an interventional radiologist during sedation. Diagnostic angiography of the limb was performed before and after the infusion (Suppl. Fig. 3).

All patients underwent muscle biopsy two months after the last infusion (Pt05 and 06 also before the first infusion) and donor chimerism was evaluated by genomic detection of patient and donor-specific short tandem repeats (STR).[22] Dystrophin expression was investigated by western blot and immunohistochemistry, including quantitative immune-fluorescence analysis, as described.[23]

MRI, FUNCTIONAL AND QUANTITATIVE MEASURES

All patients underwent periodic MRI of the hips and lower limbs (Suppl. Fig. 1) and brain MRI at the end of the scheduled treatments. All MRI examinations were performed on a 3 Tesla scanner (Philips) without sedation.

Outcome measures to test muscle strength, functional mobility, endurance, and ability to walk included the 6-minute walk test (6MWT), North Star Ambulatory Assessment (NSAA), ten meter walk, and time to rise from floor (Gower's sign), as well as quantitative functional analysis on the Kin Com ergometer.[20,24]

IMMUNOLOGICAL ANALYSIS

T-cell dynamics, alloreactive T-cell responses to donor cells and both cellular and humoral specific response to the dystrophin protein were longitudinally evaluated. Patient samples were harvested before treatment and bimonthly during MAB infusions (suppl. Material).

RESULTS

INTRARTERIAL MAB INFUSIONS

MAB infusions were safe; no SAE were observed except for Pt 03 (thalamic stroke). Details regarding adverse events are summarized below and detailed in supplementary material (Suppl. Table 2).

In pt 01 and 03 cell dose was inferior to target. Immediately after MAB infusion, an asymptomatic cutaneous reticulum (*livedo reticularis*) appeared in the left abdominal lower quadrant (Fig. 1A) in Pt 01 and disappeared spontaneously after one day; it was attributed to the infusion of small cell clumps, occasionally appearing in confluent cultures (Fig. 1B). Also Pt 02 showed a transient *livedo reticularis* after two MAB infusions (in left hand and left limb; Fig 1C and not shown). To avoid this complication we amended the protocol to allow filtration of the MP with a 70 µm cell strainer.

During the first MAB infusion, the pre-infusion diagnostic angiography of the right

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lower limb of Pt 03 revealed contrast inflow delay, likely due to vasospasm of the ipsilateral iliac-femoral arterial axis and the patient was thus infused on the contralateral, patent artery after iliac crossing; the vasospasm resolved after injection of vasodilator. Pt 03 showed one SAE after the fourth (last) infusion. He presented atrial fibrillation after MAB infusion that lasted up to 6 hours and resolved spontaneously one hour after having being detected. ECG, echocardiography and Color-Doppler Ultrasound of arteries at four limbs were all normal. The subsequent night, he had headache, photophobia and vomiting, which solved with paracetamol. Neurological examination was normal but brain MRI showed a small acute thalamic stroke (Fig 1D). Intracranial arterial and venous MR angiography (MRA) and contrast-enhanced MRA of the supra-aortic arteries showed normal caliber and flow signal of the examined vessels. Transcranial Doppler ultrasound with micro-bubbles was normal. He was started on oral aspirin and no further complication occurred. Cerebral MRI one month later showed normal evolution of the ischemic lesion (Fig. 1E). No new lesions or any clinical consequences were detected.

Due to the stroke in Pt 03, study Data Safety Monitoring Board (DSMB) recommended in Pt 05 and 06 MAB infusions only in lower limbs for safety and with the intention to increase cell dose to reach target treatment in lower limbs. No significant SAE were observed.

DONOR CELL ENGRAFTMENT AND DYSTROHIN EXPRESSION

Muscle biopsies showed histological features of muscular dystrophy in all patients (Suppl. Fig. 4), modest fiber regeneration (identified by anti-fetal myosin), ranging from 3 to 32% as compared to those usually observed in younger DMD patients (50-60%) (Suppl. Fig. 4C). DNA chimerism analysis revealed donor cell engraftment ranging from 0.00 to 0.69% (Suppl. Table 3),

Pt 01 and 03 showed virtually no dystrophin expression by immunohistochemistry (Fig. 2A and not shown). Pt 02 showed scattered, faint, dystrophin positivity in some muscle fibers in post-treatment biopsies. Fiber staining was discontinuous, but revealed also with anti-dys1 antibody, which recognizes a portion of deleted protein absent in revertant fibers (Fig. 2B). Pt 05 and 06 showed some fibers positive for dystrophin in both pre and post-treatment samples (Fig. 2C, 2D). However, in Pt 05 dystrophin expression in post-treatment was more diffuse and intense as compared to pre-treatment samples. We then applied semi-quantitative measurement of dystrophin expression levels comparing pre-treatment muscle of Pt 01, 05 and 06 (those with available pre-treatment biopsy) with levels in post-treatment muscle. Only Pt 05 showed post-treatment increase of dystrophin levels with anti-dys2 antibody, as mean dystrophin fluorescence intensity increased from 3% to 11% of normal control after treatment. Pt 01 and 06 did not show increase of protein expression (Fig. 2C, 2D and Suppl. Fig. 4D). However, similar quantification with anti-dys1 antibody did not show increase in dystrophin levels in Pt 01 and 05, whereas a moderate increase was observed in Pt 06 (from 11% to 22%).

Western blot analysis did not show any band corresponding to dystrophin (427 kD) in Pt 01 (Fig. 2E) and Pt 03 (data not shown). Pt 02 did not show any dystrophin in pre-treatment sample whereas a faint band corresponding to full-length dystrophin was observed with anti-mandys18 and manex46e only in post-treatment samples (Fig. 2F). Interestingly, mandys18 recognizes peptides from exon 17-35, deleted in Pt 02 (deletion 4-44), thus not present in revertant fibers. Pt 05 and 06 (point mutations) showed bands corresponding to dystrophin in both pre- and post-treatment samples, detected only after protein concentration with Amicon Ultra-0.5 centrifugal filter devices (Millipore) Fig. 2G, 2H). The amount of protein did not differ in pre to post-treatment sample in Pt 06, whereas it was markedly increased in post-treatment sample in Pt 05 (Fig. 2G). Since Pt 02 has a deletion of 40 exons,

the faint band of full molecular weight detected by western blot may only derive from donor (MAB) cells. In the case of Pt 05 and 06, the presence of a point mutation, makes it impossible to distinguish between donor and revertant dystrophin by western blot analysis. For this reason we conducted Sanger sequencing of Pt 05 muscle cDNA, which only detected mutated cDNA (Suppl. Fig. 5). To increase sensitivity, we performed next generation sequencing and a tetra-primer PCR assay (Suppl. Fig. 5) to detect the polymorphism. However, also these sensitive methods failed to detect donor cDNA but also ruled out skipping of the mutated allele, which was still present as the totality of the sample.

MRI OF THE LOWER LIMBS

In all MRI examinations, no signs of ischemic muscle damage, abnormal muscle edema or ectopic/expansive lesions were detected. Signal Intensity Ratio (SIR) and muscle segmental volumes indexes (MVIs) obtained from the post-processing analyses of hip and lower limb muscles of transplanted patients were compared to those obtained with 85 MRI acquisitions performed on 26 untreated DMD patients who underwent a natural history study (DMD01).[20] Four to five pre-treatment MRI exams were available also for DMD transplanted patients (Fig. 3 and Suppl. Fig 6). In Pt 05, a significant post-transplant amelioration of the trend of MRI parameters was observed for SIRs of all analyzed thigh muscles (quadriceps, thigh flexor muscles, gracilis, sartorius) and for MVIs of quadriceps and semitendinosus (Fig. 3); MVIs of soleus, biceps and gracilis muscles did not reach a significant modification of the trend though a detectable improvement was observed. After treatment in Pt 06, SIRs and MVI remained relatively stable and above the 95° percentile of the disease natural history cohort (Fig 3B and Suppl. Fig 6), but no significant modification of the trend was detected. No improvement was observed in Pt 01, 02, 03.

CLINICAL PROGRESSION OF THE DISEASE

All patients complied well with MAB infusions schedule and performed all planned functional and clinical tests. Respiratory and cardiac functions remained stable in all patients throughout the period of transplantation (Table 1). Pt 01 and 06 were already wheelchair bound and their motility could not be analyzed by functional tests. Pt 02 and 03, although ambulant, could not perform Gowers' test already before infusions. Pt 02, who was undergoing a rapid deterioration of motor functions, appeared to stabilize, following cell infusions from April 2011 to August 2012, when he grew in height and weight, begun to limp and fall spontaneously and within five months became wheelchair bound. Pt 03, progressively worsened in his quantitative and functional measures despite MAB infusions and in December 2011 lost ambulation. Finally, Pt 05 who was stable at the onset of the infusions (November 2012) is still stable at the time of writing (December 2014; Figure 4 and Suppl. Fig. 7). Quantitative measures with Kin Com ergometer did not show significant changes after cell infusions in all the patients except for Pt05. This patient showed a decrease in the slope of change of all the Kin Com parameters after the infusion, as compared to the slope from the age of 7.5 years (Suppl. Figure 8). Creatine kinase levels did not show any dose-infusion correlation in all 5 patients. Based on these results, immunosuppression with tacrolimus was gradually tapered and discontinued in all but Pt 05.

IMMUNOLOGICAL STUDIES

T lymphocytes counts and relative subsets remained stable and within normal ranges throughout the study (Suppl. Fig. 9)[25]. Accordingly, no infectious SAE were recorded. Only Pt 03 showed a high basal alloreactive response to donor MABs before treatment and this response increased after infusions. Allo-reactivity to dystrophin[26], evaluated with a dystrophin peptide library was either undetectable or very low (Pt 06). No circulating antibodies to dystrophin were detected (Suppl. Fig. 10).

DISCUSSION

We report the results of a proof-of-concept phase I/IIa trial consisting of multiple intra-arterial infusions of HLA-matched donor MABs in five DMD patients. We demonstrate that it is safe to inject hundreds of millions of non-hematopoietic stem cells into the peripheral arterial circulation of pediatric patients, a procedure never reported previously in humans. This by itself has major implications for a number of different trials: currently there are approximately 400 clinical trials, mostly based upon intra-venous administration of allogeneic mesenchymal stem cells (MSC) for a variety of disorders.[27] However the vast majority of injected cells will be trapped in the lung,[28] as the first capillary filter, whereas an intra-arterial administration of these cells may direct them to the specific anatomical district favoring their engraftment and eventual function.

Only one SAE out of 23 infusions, a thalamic stroke, occurred in Pt 03 after the last infusion. The cause of the thalamic stroke remains unclear. We excluded artery dissection and embolism from patent foramen ovale. Stroke may have been caused by a cardio-embolic event, most common mechanism of cerebral infarction described in DMD patients, although relative rare.[29] Atrial fibrillation most likely followed the stress related to the procedure and to anesthesia in a DMD patient with initial signs of cardiac involvement (sinus tachycardia 92 bpm at rest).[30] Other possibilities include i) migraine,[31] associated to 1-14% of stroke in children,[32,33] ii) minimal endothelial damage during intra-arterial catheterism, resulting in a small thrombus that, when detached hours later caused the thalamic stroke; iii): a small amount of MABs injected in the axillary artery due to whirlpool might have entered in the right vertebral artery, although this is unlikely due to the relevant distance between the catheter tip (in axillary artery) and the origin of the vertebral artery. Moreover, post-infusion arteriography never showed stop of staining due to thrombotic events by MAB injected in high number (hundreds of millions of cells) in the four limbs. No MRI-detectable adverse events (inflammation, tumor formation, tissue infarction) occurred despite the high number of cell infused.

The treatment did not approach the threshold of clinical efficacy. Nevertheless, we recorded some clinical and biological positive indications of MABs efficacy in the two youngest DMD patients. Families and physiotherapists reported less frequent falls, less rigid muscles and a more secure ambulation. Moreover, functional measures remained stable in Pt 02 (who was rapidly worsening before the infusions) and in Pt 05, who was stable throughout the study. Pt 05 also showed a decrease in the slope of changes of strength in quantitative measures by the KinCom ergometer, and a significant amelioration of the trend of MRI parameters. MRI analysis remained stable in Pt 06 and confirmed the advanced phase progression of the disease in others.

STR chimerism analysis revealed maximum donor cells engraftment of 0.69%, which is consistent with a billion MAB cells delivered to an approximately 10 Kg mass of tissue and that pre-clinical work indicated that about 30% of donor cells persist in the injected leg when measured after a week.[15,16]

Histology, immunocytochemistry, western blot and molecular analysis of the muscle biopsies performed after the treatment indicated variable but overall modest engraftment. Pt 01 has severe damaged muscle and no dystrophin indicating a clearly very advanced disease. Pt 02 was the only to show unequivocal presence of donor dystrophin detected both by antibodies directed against the deleted domain and by a full molecular weight protein that he could not possibly synthesize with a 40 exon deletion. Pt 03 did not show evidence of donor-derived dystrophin. For Pt 05 and 06 the situation was clearly more complicated due to the

presence of a point mutation. Pt 06 had a well detectable dystrophin both before and after treatment, whereas Pt 05 only after. To rule out the possibility that it was due to revertant fibers, indistinguishable by western blot, we performed sophisticated molecular analysis that unexpectedly revealed that all the dystrophin cDNA detected has the expected point mutation and thus comes from the patient. However, it is not derived from spontaneous exon skipping, as the exon containing the mutation was still present as the only transcript detected. We speculate that some patients (e.g. 05 and 06) may have a PTC124-like endogenous mechanism that allows the production of some "full-length" dystrophin, as shown for other human genes.[34] Intriguingly, Pt 06 is the strongest (though unable to walk because very tall) and Pt 05 is still walking at the time of writing. However, given the paucity of donor MAB engraftment, a transient paracrine effect is possible, similar to that elicited by transplanted MSC.

Immunological analysis revealed that immune suppression did not alter lymphocyte counts or their in vitro activity, that the only cell mediated response to donor cells was observed in Pt 03, and was detectable before MAB infusion, suggesting that this immunosuppressive regimen is adequate to prevent primary responses, but is not active on memory immunological responses. All patients had undetectable or extremely low responses against dystrophin domains, including those absent because of large deletions.

In conclusion, our study was safe and provided relevant information essential for planning future cell therapy protocols in DMD patients. The adopted schedule and doses of MAB infusions did not reach efficacy in patients at late age at the time of treatment. Further trials will target younger patients, thus taking advantage of a less advanced pathology and a higher cell dose in patients before the onset of symptoms. In parallel, a cell-mediated gene correction strategy (e.g. artificial chromosomes, genome editing) that may amplify several fold the therapeutic effect and different angiographic strategies to target pelvic and dorsal muscles will be tested in animal models. We are confident that the final combination of these strategies may lead to clinical efficacy in DMD correction.

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Table 1: Patient clinical features:

	Pt 01	Pt 02	Pt 03	Pt 05	Pt 06
Dystrophin mutations	Exon deletion 47-52	Exon deletion 4-44	Exon deletion 45-50	Point mutation exon 43	Point mutation exon 59
Steroid (mg/kg)	Deflazacort 0.75 / every other day	Deflazacort 0.7 / every other day	Prednisone 0.5/ daily	Deflazacort 0.9 / every other day	Deflazacort 0.5 / every other day
Age at first infusion (yr)	12.4	8.5	9.6	9.2	12.2
Loss of ambulation (age)	12	10.2	10. 8	Preserved walk ability	12.1

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Weight (kg)*	50	25	37	38	46
Height (cm)*	137	123	140	126	160
Cardiac function	Normal	Normal	Normal	Normal	Normal
Lung function	Normal	Normal	Normal	Normal	Normal
Other co-morbidities	None	None	None	None	None

Legend: *Measured at first infusion.

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FIGURE LEGENDS

Figure 1: Side effects of MAB treatment of DMD patients.

Panel A: the *livedo reticularis* in the left abdominal lower quadrant after the first infusion in Pt 01.

Panel B: small clump of MABs observed in the first preparation of MP before the infusion of Pt 01.

Panel C: the *livedo reticularis* in the left hand of Pt02 after the first infusion.

Panel D: brain MRI acquired one day after the MAB infusion showing acute small thalamic stroke in Pt03. Axial diffusion-weighted imaging (left) and fluid-attenuated inversion recovery (FLAIR; right) images show a focal spot of hyperintensity within the right thalamus consistent with acute stroke.

Panel E: FLAIR MRI axial image obtained in Pt 03 one month after the acute stroke, showing the expected evolution of the right thalamic lesion.

Figure 2: Effects of MAB treatment on dystrophin expression.

Panel A: confocal immunofluorescence of muscle biopsy from Pt 01 (tibialis anterior post-treatment) stained with anti-dystrophin dys1 antibody (which recognizes protein fragment encoded by exons 26-30, green signal) and anti laminin-2 (to delineate muscle fibers, red signal); dapi identifies nuclei (blue signal). In the left image only anti dystrophin staining is shown. No dystrophin positive fibers were observed.

Panel B: confocal immunofluorescence of muscle biopsy from Pt 02 (tibialis anterior post-treatment) stained with anti-dystrophin dys1 antibody (green signal) and anti lamininin-2 (red signal); dapi identifies nuclei (blue signal). In the left image only anti dystrophin staining is shown. Some fiber shows mild and discontinuous dystrophin staining.

Panel C: immunofluorescence of muscle biopsy from Pt 05 taken before (left, gastrocnemius) and after treatment (right, gastrocnemius) stained with anti dystrophin dys2 antibody (which recognizes exon 77-79). The number and intensity of dystrophin-positive fibers is increased in the post-treatment biopsy.

Panel D: immunofluorescence of muscle biopsy from Pt 06 taken before (left, gastrocnemius) and after treatment (right, gastrocnemius) stained with anti dystrophin dys2 antibody. Few fibers show scattered dystrophin staining, without obvious differences between pre and post-treatment samples.

Panel E: results of western blot analysis involving dystrophin antibodies dys1, Mandys18 and Manex46e (recognizing respectively exon 26-30, 17-35, and 46), of total protein extracts (20 µg) obtained from post-treatment biopsy specimens of Pt 01 (tibialis anterior muscle); Ct was used as positive dystrophin control. Below, bands corresponding to myosin heavy chain are shown as loading control. No bands corresponding to full length dystrophin were observed. A schematic representation of the deleted portion of the dystrophin and the region recognized by used antibodies is depicted above the western blot.

Panel F: results of western blot analysis involving dystrophin antibodies Mandys18 and Manex46e, of total protein extracts (10-20 µg) obtained from pre-treatment (performed at time of diagnosis) and post-treatment biopsy specimens of Pt 02 (tibialis anterior muscle); Ct was used as positive dystrophin control. Below, bands corresponding to myosin heavy chain are shown as loading control. One faint band corresponding to full length dystrophin is observed only in the post-treatment samples with both Mandys18 and Manex46e antibodies. A schematic representation of the deleted portion of the dystrophin and the region recognized by used antibodies is depicted above the western blot.

Panel G: results of western blot analysis involving dystrophin antibodies Mandys106 (recognizing exon 43) and dys1, of total protein extracts (80 µg, following protein

concentration by Amicon Ultra-0.5 centrifugal filter devices, Millipore) obtained from pre-treatment and post-treatment biopsy specimens of Pt 05 (gastrocnemius); Ct was used as positive dystrophin control. Below, bands corresponding to myosin heavy chain are shown as loading control. Bands corresponding approximately to full length dystrophin are observed in pre and post-treatment samples with both antibodies However, bands in post-treatment sample appeared higher in amount.
Panel H: results of western blot analysis involving dystrophin antibodies Mandys106 and dys1, of total protein extracts (80 µg, following protein concentration by Amicon Ultra-0.5 centrifugal filter devices, Millipore) obtained from pre-treatment and post-treatment biopsy specimens of Pt 06 (gastrocnemius); Ct was used as positive dystrophin control. Below, bands corresponding to myosin heavy chain are shown as loading control. Bands corresponding approximately to full length dystrophin are observed in pre and post-treatment samples with both antibodies Bands in pre-treatment sample appeared higher in amount as compared to post-treatment sample.

Figure 3: Effects of MAB treatment on MRI of lower limbs.
Panel A: Axial T1-weighted images of the right thighs obtained in patients immediately before (upper panel) and 18 months after MAB infusion. Please note the progression of fatty degeneration and atrophy except for Pt 05 where no evident modifications can be detected.
Panel B: representative percentile of MRI quantitative parameters in transplanted patients compared to untreated patients. SIR (left image) and MVI (right image) of the quadriceps. Red line: Pt 01; dotted green line: Pt 02; dotted purple line: Pt 03; dotted dark blue line: Pt 05; dotted light blue line: Pt 06). Empty dots correspond to pre-treatment measures. Orange dots correspond to post-transplantation measurement. The black line corresponds to the median, while gray dotted lines to 75°, 90° and 95° percentile. Please note the modification of the trend of Pt 05 after treatment.
Panel C: representative quantile spline regressions of post-transplant trend amelioration of MRI quantitative parameters in Pt 05. Upper left: SIR of the quadriceps. Lower left: SIR of thigh flexor muscles. Upper right: MVI of the quadriceps. Lower right: MVI of the semitendinosus muscles. Please note the significant modification of the patient trend (red lines) compared to median measurements obtained with 85 MRI examinations of untreated patients. Empty dots correspond to pre-treatment measures. Full dots correspond to post-transplantation measurement. The grey dotted line corresponds to the time of the first intra-arterial transplantation.

Figure 4: Effects of MAB treatment on outcome measures of DMD patients.
Panel A: North Star scale measurement (NSAA score) plotted against age of ambulant DMD patients before, throughout and after MAB clinical trial. Arrows indicate time points of MAB infusion. Pt02 and Pt05 showed score stabilization throughout 8 months of MAB infusions; Pt05 showed stabilization even in the subsequent period, whereas Pt02 showed progressive deterioration until lost of ambulation. Pt03 showed progressive deterioration throughout MAB infusion and lost ambulation soon after the end of the trial.
Panel B: six minute walking test (meters) plotted against age of ambulant DMD patients before, throughout and after MAB clinical trial. Arrows indicate time points of MAB infusion. Pt02 and Pt05 showed score stabilization throughout 8 months of MAB infusions; Pt05 showed stabilization even in the subsequent period, whereas Pt02 showed progressive deterioration until lost of ambulation. Pt03 showed progressive deterioration throughout MAB infusion and lost ambulation soon after the end of the trial.
Panel C: time to run 10 meters (time) plotted against age of ambulant DMD patients before, throughout and after MAB clinical trial. Arrows indicate time points of MAB infusion. Pt02

and Pt05 showed time stabilization throughout 8 months of MAB infusions; Pt05 showed stabilization even in the subsequent period, whereas Pt02 showed progressive increase of time until lost of ambulation. Pt03 showed progressive increase of time throughout MAB infusion and lost ambulation soon after the end of the trial.
Panel D: time to perform Gowers' maneuver (seconds) plotted against age of ambulant DMD patients before, throughout, and after MAB clinical trial. Arrows indicate time points of MAB infusion. Pt 02 and Pt 06 were already unable to perform the exercise at the onset of the clinical trial. Pt 05 showed stabilization of the test during the clinical trial.

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figure 1

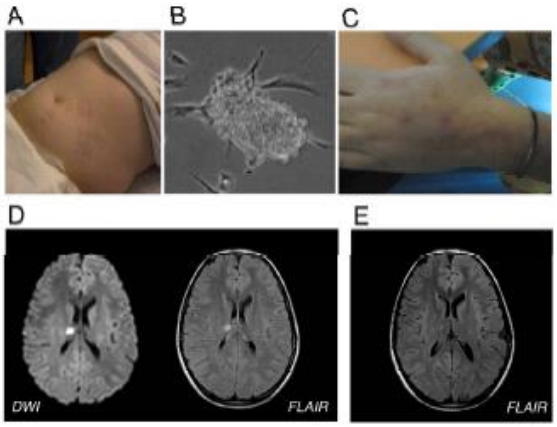

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Figure 2

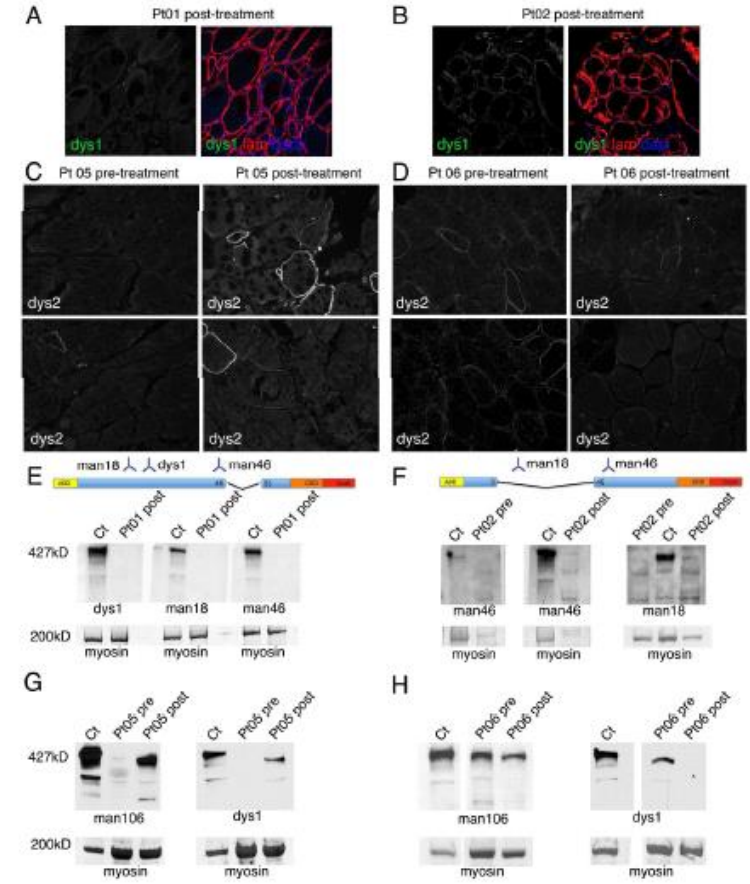

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Figure 3

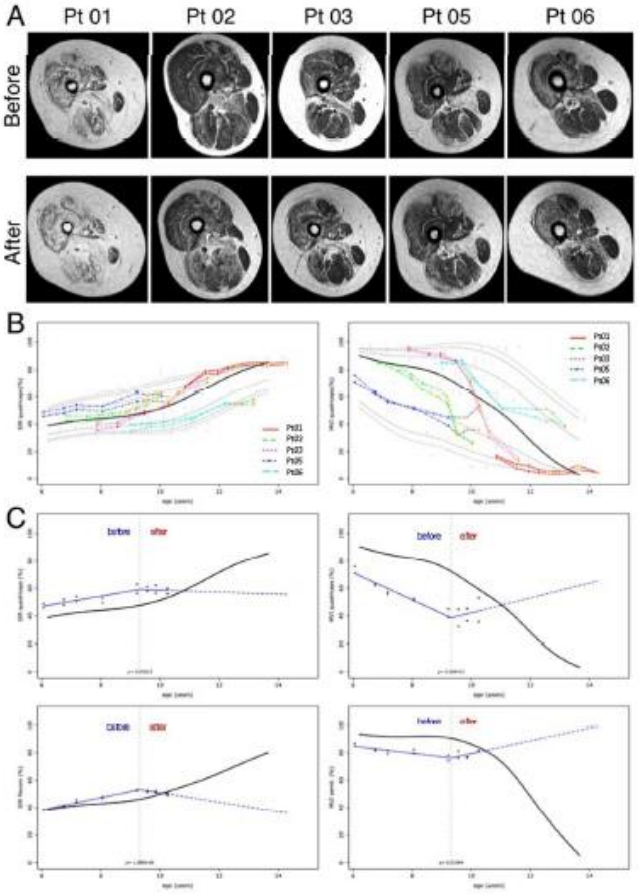

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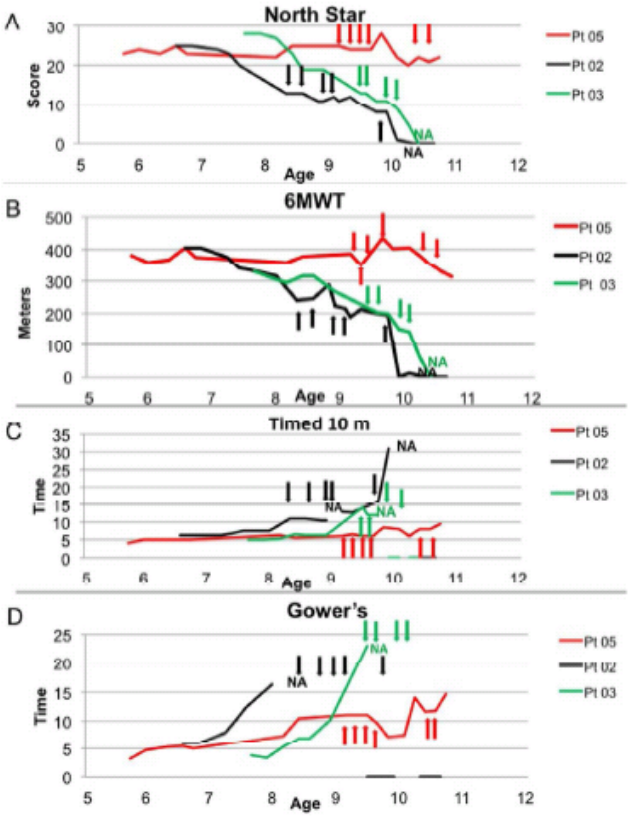
Figure 4



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Chapter 3

“Preliminary results on phase I/II clinical trial of haematopoietic stem cell gene therapy for the Wiskott-Aldrich Syndrome”

RESEARCH ARTICLE SUMMARY

Lentiviral Hematopoietic Stem Cell Gene Therapy in Patients with Wiskott-Aldrich Syndrome

Alessandro Aiuti,* Luca Biasco, Samantha Scaramuzza, Francesca Ferrua, Maria Pia Cicalese, Cristina Baricordi, Francesca Dionisio, Andrea Calabria, Stefania Giannelli, Maria Carmina Castiello, Marita Bosticardo, Costanza Evangelio, Andrea Assanelli, Miriam Casiraghi, Sara Di Nunzio, Luciano Callegaro, Claudia Benati, Paolo Rizzardi, Danilo Pellin, Clelia Di Serio, Manfred Schmidt, Christof Von Kalle, Jason Gardner, Nalini Mehta, Victor Neduva, David J. Dow, Anne Galy, Roberto Miniero, Andrea Finocchi, Ayse Metin, Pinaki P. Banerjee, Jordan S. Orange, Stefania Galimberti, Maria Grazia Valsecchi, Alessandra Biffi, Eugenio Montini, Anna Villa, Fabio Ciceri, Maria Grazia Roncarolo, Luigi Naldini

READ THE FULL ARTICLE ONLINE
http://dx.doi.org/10.1126/science.1233151

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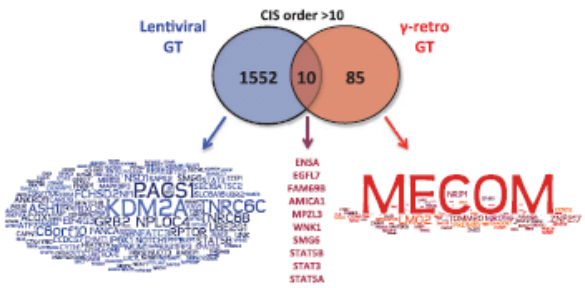
Introduction: Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency characterized by eczema, thrombocytopenia, infections, and a high risk of developing autoimmunity and cancer. In a recent clinical trial, a γ-retroviral vector was used to introduce a functional WAS gene into autologous hematopoietic stem/progenitor cells (HSCs), followed by reinfusion of the gene-corrected HSCs into the patients. This strategy provided clinical benefit but resulted in expansion and malignant transformation of hematopoietic clones carrying vector insertions near oncogenes, thus increasing leukemia risk. We have developed a clinical protocol for WAS based on lentiviral vector (LV) gene transfer into HSCs.

Methods: Three patients with WAS were treated in a phase I/II clinical trial with gene-corrected HSCs after pretreatment with a reduced-intensity myeloablative regimen. Autologous CD34$^+$ cells were transduced with an optimized LV carrying the WAS gene under the control of its endogenous promoter. Patients were monitored for up to 2.5 years after gene therapy by molecular, immunological, and clinical tests. We also investigated the genomic distribution of LV integration sites in the patients' bone marrow and peripheral blood cell lineages.

Results: Administration of autologous HSCs transduced with LV at high efficiency (>90%) resulted in robust (25 to 50%), stable, and long-term engraftment of gene-corrected HSCs in the patients' bone marrow. WAS protein expression was detected in myeloid cells at similar rates and in nearly all circulating platelets and lymphoid cells. In vitro T cell proliferative responses, natural killer cell cytotoxic activity, immune synapsis formation, and suppressive function of T regulatory cells were normalized. In all three patients, we observed improved platelet counts, protection from bleeding and severe infections, and resolution of eczema. Vector integration analyses on >35,000 unique insertion sites showed distinct waves of HSC clonal output, resulting in highly polyclonal multilineage hematopoietic reconstitution. In contrast to γ-retroviral gene therapy, our LV-based therapy did not induce in vivo selection of clones carrying integrations near oncogenes. Consistent with this, we did not see evidence of clonal expansions in the patients for up to 20 to 32 months after gene therapy.

Discussion: Our gene transfer protocol provided efficient stem cell transduction in vitro, resulting in robust and stable in vivo gene marking. WAS expression was restored to near-physiological levels in the patients, resulting in immunological and hematological improvement and clinical benefit. Clonal tracking of stem cell dynamics by vector insertions showed details of hematopoietic reconstitution after gene therapy. Comparison with clinical data from γ-retroviral gene therapy in the same disease setting strongly suggests that LV gene therapy offers safety advantages, but a longer follow-up time is needed for validation. Collectively, our findings support the use of LV gene therapy to treat patients with WAS and other hematological disorders.



Analysis of common insertion sites (CIS) in gene therapy trials using lentiviral versus γ-retroviral vectors. Word clouds show the intensity of insertion sites clustering in each of the CIS genes (the larger the gene name, the larger the number of insertion sites within or in the proximity of that gene). The names of the CIS genes detected in both gene therapy trials are reported at the intersection between the circles.

FIGURES AND TABLE IN THE FULL ARTICLE

SUPPLEMENTARY MATERIALS

Supplementary Text
Figs. S1 to S46
Tables S1 to S25
References

RELATED ITEMS IN *SCIENCE*

A. Biffi *et al.*, Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* **341**, 1233158 (2013); doi:10.1126/science.1233158

I. M. Verma, Edging toward the promise of gene therapy. *Science* **341**, 853–855 (2013); doi:10.1126/science.1242551

The list of author affiliations is available in the full article online.
*Corresponding author. E-mail: aiuti.alessandro@hsr.it

RESEARCH ARTICLE

Lentiviral Hematopoietic Stem Cell Gene Therapy in Patients with Wiskott-Aldrich Syndrome

Alessandro Aiuti,[1,2,3,4]* Luca Biasco,[1]† Samantha Scaramuzza,[1]† Francesca Ferrua,[2,3,5] Maria Pia Cicalese,[2,3] Cristina Baricordi,[1] Francesca Dionisio,[1] Andrea Calabria,[1] Stefania Giannelli,[1] Maria Carmina Castiello,[1,5] Marita Bosticardo,[1] Costanza Evangelio,[2,3] Andrea Assanelli,[3,6] Miriam Casiraghi,[2] Sara Di Nunzio,[2] Luciano Callegaro,[2] Claudia Benati,[7] Paolo Rizzardi,[7] Danilo Pellin,[8] Clelia Di Serio,[8] Manfred Schmidt,[9] Christof Von Kalle,[9] Jason Gardner,[10] Nalini Mehta,[11] Victor Neduva,[11] David J. Dow,[11] Anne Galy,[12] Roberto Miniero,[13] Andrea Finocchi,[4] Ayse Metin,[14] Pinaki P. Banerjee,[15] Jordan S. Orange,[15] Stefania Galimberti,[16] Maria Grazia Valsecchi,[16] Alessandra Biffi,[1,2,3] Eugenio Montini,[1] Anna Villa,[1,17] Fabio Ciceri,[3,6] Maria Grazia Roncarolo,[1,2,3,5]‡ Luigi Naldini[1,5]‡

Wiskott-Aldrich syndrome (WAS) is an inherited immunodeficiency caused by mutations in the gene encoding WASP, a protein regulating the cytoskeleton. Hematopoietic stem/progenitor cell (HSPC) transplants can be curative, but, when matched donors are unavailable, infusion of autologous HSPCs modified ex vivo by gene therapy is an alternative approach. We used a lentiviral vector encoding functional WASP to genetically correct HSPCs from three WAS patients and reinfused the cells after a reduced-intensity conditioning regimen. All three patients showed stable engraftment of WASP-expressing cells and improvements in platelet counts, immune functions, and clinical scores. Vector integration analyses revealed highly polyclonal and multilineage haematopoiesis resulting from the gene-corrected HSPCs. Lentiviral gene therapy did not induce selection of integrations near oncogenes, and no aberrant clonal expansion was observed after 20 to 32 months. Although extended clinical observation is required to establish long-term safety, lentiviral gene therapy represents a promising treatment for WAS.

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency characterized by infections, microthrombocytopenia, eczema, autoimmunity, and lymphoid malignancies (*1*, *2*). The disorder is caused by mutations in the *WAS* gene, which codes for WASP, a protein that regulates the cytoskeleton. WASP-defective immune cells display alterations in proliferative responses after activation, cell migration, immunological synapsis formation, and cytotoxicity (*3*–*5*). Allogeneic hematopoietic stem/progenitor cell (HSPC) transplantation can be curative, but it is often associated with substantial morbidity and mortality, particularly in the absence of fully matched donors (*6*–*8*).

For patients without matched donors, an alternative therapeutic strategy is the infusion of autologous HSPCs that have been genetically corrected ex vivo. This gene therapy approach has been successful in more than 50 patients affected by primary immunodeficiencies, including 10 WAS patients treated with HSPCs transduced with a γ-retroviral vector encoding a functional WAS gene (*9*–*15*). Gene therapy combined with a reduced intensity conditioning regimen proved to be effective and safe in patients with severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA) deficiency, who were followed up to 13 years after treatment (*9*, *15*, *16*). In contrast, despite the initial clinical benefit, gene therapy with γ-retroviral–transduced HSPC was associated with the development of leukemia or myelodysplasia in patients with SCID-X1, chronic granulomatosis disease, and WAS (*14*, *17*–*20*). These adverse events were ascribed to vector insertion sites (ISs) near specific proto-oncogenes, leading to their transactivation by enhancer/promoter sequences within the long-terminal repeat (LTR) of the retroviral vector (*10*–*12*, *21*–*23*). In the case of WAS, characterization of ISs over the first 2 years of follow-up revealed a highly skewed insertion profile in vivo, resulting in the expansion of clones with insertions in proto-oncogenes such as *LMO2* (*12*), some of which progressed to leukemias (*14*, *24*). The possibility of vector-driven leukemogenesis is a particular concern for WAS patients, who are cancer-prone (*1*).

Lentiviral vectors with self-inactivating (SIN) LTRs integrate efficiently in HSPC, allow robust transgene expression from a promoter of choice inserted within the vector, and could potentially be safer for gene therapy applications (*24*–*26*). Lentiviral-based HSPC gene therapy combined with full conditioning has been used to treat three patients with adrenoleukodystrophy (ALD) (*27*) and one patient with β-thalassemia (*28*), resulting in 10 to 15% progenitor cell marking with therapeutic benefit. Although a relative expansion of a clone harboring an insertion in the *HMGA2* gene was observed in the β-thalassemia patient (*28*), no aberrant clonal proliferation has been reported for the lentiviral-based trials up to 5 years after treatment (*27*, *29*).

We developed a SIN lentiviral vector coding for human WASP under the control of a 1.6-kb reconstituted WAS gene promoter (LV-w1.6W) (*3*). The use of this endogenous promoter ensures that the transgene is expressed in a physiological manner (*4*), restoring WASP expression and function in human and murine WAS cells (*3*, *30*–*34*). Its moderate enhancer activity combined with the SIN LTR design reduces the risk of insertional mutagenesis (*35*), as shown by in vitro transformation assays (*36*) and preclinical in vivo studies in WASP-deficient mice (*34*, *37*). These data provided the rationale for a phase I/II clinical trial in which LV-w1.6W was used as a gene therapy vector for treatment of patients with WAS (*38*).

Results

Lentiviral Transduction of HSPC and Infusion of Gene-Corrected Cells into Patients Pretreated with Reduced Intensity Conditioning

Three children with WAS, who had been shown by genotyping to carry severe mutations in the X-linked *WAS* gene and who did not have compatible allogeneic donors, were enrolled in the phase I/II clinical trial (Table 1). All patients suffered from recurrent infections, eczema, bleeding, and thrombocytopenia, with a disease score ranging from 3 to 4 (*39*) (Table 1). Autologous bone-marrow (BM)–derived $CD34^+$ cells were collected, transduced twice with purified LV-w1.6W vector using an optimized protocol (fig. S1) (*34*), and reinfused intravenously back into the patients 3 days after collection. The vector and genetically modified

[1]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells, and Gene Therapy, San Raffaele Scientific Institute, 20132 Milan, Italy. [2]HSR-TIGET Pediatric Clinical Research Unit, San Raffaele Scientific Institute, 20132 Milan, Italy. [3]Pediatric Immunohematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, 20132 Milan, Italy. [4]University Department of Pediatrics (DPUO), Bambino Gesù Children's Hospital and Tor Vergata University, School of Medicine, 00165 Rome. [5]Vita-Salute San Raffaele University, 20132 Milan, Italy. [6]Hematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, 20132 Milan, Italy. [7]MolMed S.p.A., 20132 Milan, Italy. [8]University Centre of Statistics in the Biomedical Sciences (CUSSB), Vita-Salute San Raffaele University, 20132 Milan, Italy. [9]Nationales Zentrum für Tumorerkrankungen (NCT), Deutsches Krebsforschungszentrum (DKFZ), 69120 Heidelberg, Germany. [10]Regenerative Medicine Discovery Performance Unit, GlaxoSmithKline Research and Development, King of Prussia, 19406 PA, USA. [11]Molecular and Cellular Technologies, GlaxoSmithKline, Stevenage Herts SG1 2NY, UK. [12]Unité Mixte de Recherche (UMR) 951, Molecular Immunology and Biotherapies, Genethon, 91002 Evry, France. [13]Dipartimento di Scienze Mediche e Chirurgiche, Università Magna Graecia di Catanzaro, 88100 Catanzaro, Italy. [14]Ankara Children's Hospital, 06050 Ankara, Turkey. [15]Texas Children's Hospital, Baylor College of Medicine, Houston, TX 77030, USA. [16]Dipartimento di Medicina Clinica e Prevenzione, University of Milano-Bicocca, 20126 Monza, Italy. [17]Istituto di Ricerca Genetica e Biomedica (IRGB)-Consiglio Nazionale delle Ricerche (CNR), 20138 Milan, Italy.

*Corresponding author. E-mail: aiuti.alessandro@hsr.it
†These authors contributed equally to this work.
‡These authors contributed equally to this work.

cells were characterized extensively for quality and safety (tables S1 to S3 and figs. S1 and S2).

Vector-specific quantitative real-time fluorescence polymerase chain reaction (qPCR) on individually collected colony-forming cells (CFC) revealed high gene transfer efficiency—i.e., 88 to 100%. Average vector copy number (VCN) per genome measured in bulk-cultured $CD34^+$ cells was 2.3 ± 0.6 (Table 1). WASP expression in the transduced $CD34^+$ cells was confirmed by immunoblotting (fig. S3). Before HSPC reinfusion, patients received a reduced-intensity conditioning regimen, consisting of monoclonal antibody (mAb) to CD20, busulfan [7.6 to 10.1 mg/Kg intravenous (i.v.), targeted for weight and pharmacokinetics], and fludarabine (60 mg/m^2), designed to achieve considerable depletion of endogenous HSPC and immune cells with limited toxicity (see Materials and Methods). The mean infused $CD34^+$ cell dose was $11.07 \pm 2.70 \times 10^6$ cells/kg (Table 1 and table S4). No adverse reactions were observed after the infusions.

Engraftment of Lentiviral-Transduced Cells and Restoration of WASP Expression

All three WAS patients showed robust and multilineage engraftment of gene-corrected cells in BM and peripheral blood (PB), persisting up to the latest time point analyzed (30 months after gene therapy). Gene-marking in PB granulocytes peaked in the first month after treatment and then stabilized at a VCN range of 0.4 to 0.9 (Fig. 1A). Similar marking levels were seen in PB monocytes (Fig. 1A), as well as in granulocytic and megakaryocytic cells, $CD34^+$ cells (Fig. 1B), and erythroid cells (fig. S4) collected from the BM. The in vivo engraftment of lentiviral-transduced HSPC was confirmed throughout the follow-up and persisted at high levels [patient 1 (Pt1), 34%; Pt2, 26%; Pt3, 48%] 1 year after treatment, as shown by vector-specific PCR on BM CFC (Fig. 1C). Based on these data, we estimated that each transduced progenitor cell contained on average 1 to 1.6 copies of the vector. Transduced B and natural killer (NK) cells were detectable 1 month after gene therapy and increased over time, with PB-derived B cells showing VCN higher by a factor of 1.5 to 2 as compared with BM B cells (Fig. 1, A and B). Transduced T cells appeared 3 to 6 months after gene therapy and reached the highest VCN as compared with other lineages (VCN range 1.0 to 2.4) (Fig. 1A).

WASP expression, as measured by flow cytometry, progressively increased over time in PB lymphocytes and platelets in all three patients (Fig. 1D). WAS protein was present in a large proportion of T cells ($CD4^+$ and $CD8^+$), NK cells, and monocytes at mean expression level comparable to normal donors, whereas in B cells and platelets its expression level was lower than in controls (fig. S5). Interestingly, the proportion of $WASP^+$ cells was higher in PB-derived platelets as compared with their BM-derived counterparts (fig. S6), suggesting a preferential migration or selective survival advantage of gene-corrected platelets in the periphery.

Clinical and Immunological Improvement After Gene Therapy

All treated children are clinically well with post-treatment follow-up of 20 to 32 months (Table 1). After conditioning, patients experienced severe neutropenia (neutrophil counts <500/μl) lasting from 12 to 19 days, followed by normalization of neutrophil counts. None of the patients experienced mucositis or other chemotherapy-related toxicity. Serious adverse events occurred in Pt2 and Pt3 within the first 2 to 6 months of gene therapy. These events were mainly of infectious origin, likely favored by the underlying clinical conditions and immune deficiency typical of the early post-transplant period. Both patients fully recovered from them (see Materials and Methods). No abnormal cellular expansion was detected in BM and PB by immunophenotypic, morphologic, and karyotypic analyses. Assays to detect antibodies to HIV Gag p24 and replication-competent lentivirus in patients' blood were negative at all times of analysis.

In all three children, symptoms of WAS showed substantial improvement. Pretreatment eczema resolved between 6 and 12 months after gene therapy and did not recur (Table 1). Beginning 6 months after gene therapy, the frequency and severity of infections progressively decreased, and cytomegalovirus replication was well-controlled, allowing withdrawal of anti-infectious prophylaxis in Pt1 and Pt3. Pt1 discontinued i.v. immunoglobulin supplementation 6 months after gene therapy, with positive antibody responses after vaccination.

Platelet counts improved significantly during the first year after gene therapy (Fig. 2A), as assessed by a mixed linear model applied to the serially repeated sampling ($P = 0.03$). Although these counts never reached normal values, the platelet volumes normalized after gene therapy (mean platelet volume range, 8.3 to 9.2 fl; normal values, 7.4 to 10.9 fl). After discontinuation of platelet transfusions (1 to 7 months after gene therapy), none of the patients experienced bleeding or skin manifestations of thrombocytopenia, with the exception of occasional grade 1 gastrointestinal bleeding in Pt2 (Fig. 2B). Moreover, all patients tested negative for a large panel of autoantibodies 1 year after gene therapy. The overall clinical improvement resulted in a reduced disease severity score in all patients (Fig. 2C).

Lymphocyte subpopulation counts decreased after the conditioning regimen but progressively

Table 1. Characteristics and treatment of the three WAS patients. WASP expression analysis was performed on PB lymphocytes by FACS. Patient 1 also received G-CSF mobilized peripheral blood (MPB)-derived $CD34^+$ cells, previously collected as back-up, to achieve the target HSPC dose. A&W, alive and well; CMV, cytomegalovirus; ENT, ear, nose, throat; GI, gastrointestinal; GE, gastroesophageal; HHV-6, human herpes virus type 6; HSV, herpes simplex virus; IVIG, intravenous immunoglobulins; URTI, upper respiratory tract infection; UTI, urinary tract infection; VZV, *Varicella zoster* virus.

	Patient 1	Patient 2	Patient 3
Infectious manifestations	Recurrent ENT	Pneumonias, colitis arthritis/cellulitis, URTI, UTI	Pneumonia with respiratory distress, URTI, otitis
Pathogens	VZV, CMV, HSV, EBV	CMV, HHV-6, candida	*Pneumocystis jirovecii*, CMV
Thrombocytopenia manifestations	Skin petechiae	Skin petechiae, GI bleeding	Skin petechiae, GI bleeding, epistaxis
Eczema	Moderate-severe	Moderate-severe	Severe
Other	Developmental disorder, allergy	Failure to thrive, elevated inflammatory indexes/vasculitis, hepatosplenomegaly	GE reflux/food aversion (fed by nasogastric tube), allergy
WAS mutation	Exon 10: C>T 995 (R321X)	IVS10del11nt	37C>T (R13X)
WASP expression	<5%	<5%	<5%
Zhu score	3	4	4
Age at treatment (years)	5.9	1.6	1.1
Infused $CD34^+$ cells ($\times 10^6$/kg)	3.66 (BM) + 5.25 (MPB)	14.1	10.2
Vector copies/genome	1.9 (BM) – 1.4 (MPB)	2.4	2.8
Transduction efficiency (CFC)	92% (BM) – 88% (MPB)	97%	100%
Follow-up (months)	32	23	20
Current clinical conditions	A&W, no eczema, no major bleeding or petechiae, off IVIG	A&W, no eczema, no major bleeding or petechiae	A&W, no eczema, no major bleeding or petechiae

returned to normal levels for age within 6 to 12 months after treatment (fig. S7). In all patients, proliferative responses to mAb to CD3 increased after treatment and reached the range of age-matched controls (Fig. 2D). Similarly, the proliferative capacity of T cell lines from Pt1 and Pt3 greatly improved after gene therapy (fig. S8). T cell receptor (TCR) Vβ profiling revealed that all three WAS patients displayed a broader polyclonal repertoire after gene therapy, in agreement with the polyclonal reconstitution observed by IS analysis (see below) (fig. S9).

Because regulatory T (T_{reg}) cell function is defective in WAS patients (*32*, *40*), we evaluated the suppressive ability of T_{reg} cells (VCN, 1.37) sorted from Pt1. As shown in Fig. 2E, the in vitro suppressive function of T_{reg} cells was restored after gene therapy. This is consistent with the finding that $CD4^+/CD25^{high}$ cells from both Pt1 and Pt3 express WASP after gene therapy (fig. S10). It is known that WASP-deficient NK cells show an altered immune function (*41*). We found that WASP-expressing, but not WASP-deficient, NK cells isolated from Pt1 after gene therapy functioned normally in immunological synapse formation (Fig. 2F and fig. S11). The improved NK cell function of all three patients resulted in normal cytotoxic activity after gene therapy (Fig. 2G).

Multilineage and Polyclonal Engraftment of Gene-Corrected HSPC, Assessed by Longitudinal Integration Site Profiling

To monitor the clonal contribution of gene-corrected cells to hematopoiesis in the three patients, we performed a high-throughput IS analysis (*42*, *43*) on multiple lineages and time points up to 18 months after infusion of transduced HSPCs (see Supplementary Text, figs. S12 to S46, and tables S8 to S25). Linear amplification-mediated (LAM) PCR and next-generation sequencing (*42*, *43*) detected >2,400,000 IS sequences that were mapped to 33,363 unique chromosomal positions in the patients: 11,137 from Pt1, 10,889 from Pt2, and 10,337 from Pt3.

The relative proportion of sequencing reads corresponding to each specific IS, as collected from BM $CD34^+$ cells and PB myeloid B and T cells, was used as a surrogate of clonal repertoire of gene-corrected cells and safety readout (Fig. 3 and figs. S12 to S14). We found that, beginning 1 month after gene therapy, the vast majority of ISs from all lineages and time points were well below 5%, with a few occasionally reaching up to 15 to 20% of the total reads retrieved from a lineage (Fig. 3A). By monitoring the most frequent ISs in each lineage, we found that highly represented insertion sites were different at each time point and fluctuated over time for up to 6 to 12 months after gene therapy (Fig. 3B). At later time points, in line with the stabilization of

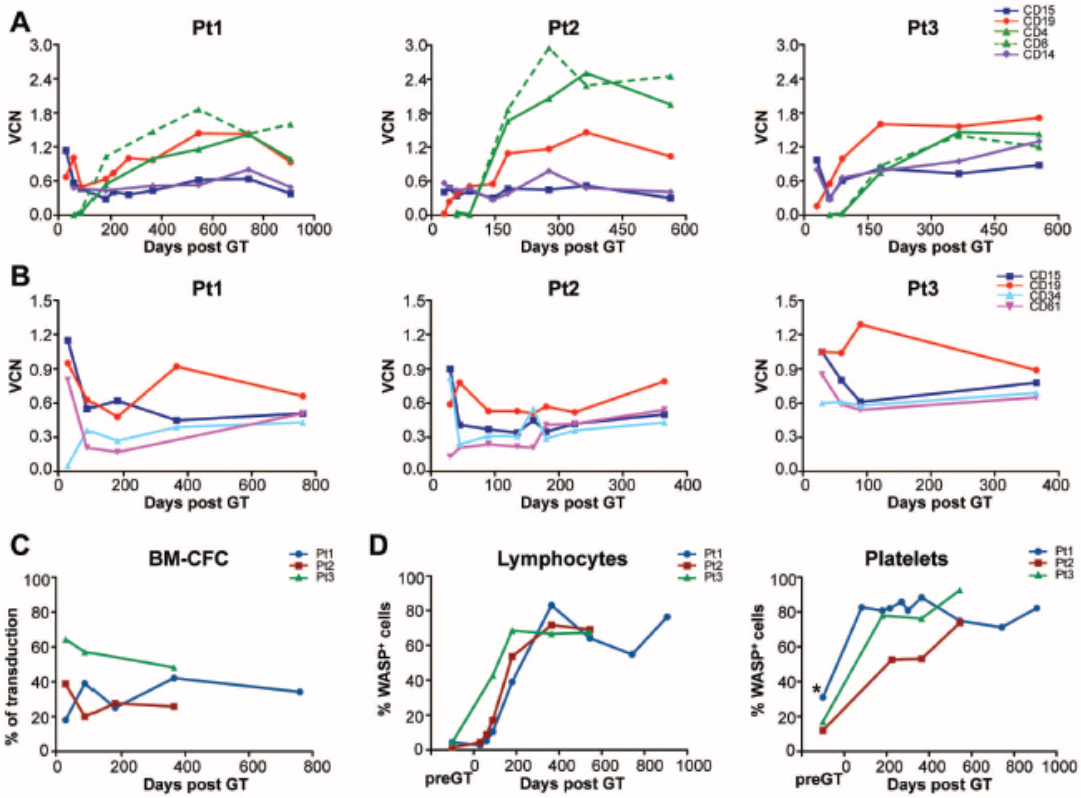


Fig. 1. Engraftment of transduced cells and WASP expression after gene therapy. VCN per genome was evaluated by qPCR at different time points (up to 2.5 years) after gene therapy (GT) in $CD15^+$ granulocytes, $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, $CD34^+$ progenitors, and $CD61^+$ (megakaryocytic lineage), purified either from peripheral blood (**A**) or bone marrow (**B**) in Pt1 (left), Pt2 (center) and Pt3 (right). (**C**) Percentage of vector-positive BM CFC evaluated by PCR analyses on individual colonies derived from ex vivo purified $CD34^+$ cells after gene therapy. (**D**) WAS protein expression measured by cytofluorimetric analysis at different time points after gene therapy in patient lymphocytes (left) and platelets (right). *, In Pt1, WASP expression was measured after transfusion of donor platelets. Transgene expression was confirmed by immunoblot analysis of peripheral blood mononuclear cells, untransformed T-cell lines, and a EBV-transformed B cell line (fig. S3).

hematopoietic output, we detected a persistent multilineage contribution of several ISs, some of which were highly represented (Fig. 3B), but remained below 10% of all the reads belonging to ISs retrieved from the PB cells in the 1-year interval (Fig. 3A and fig. S14). It is possible that HSPC activity in Pt2 was affected by the occurrence of bacterial sepsis 6 months after gene therapy. At that time, a group of ISs marking T cells, B cells, and myeloid cells became relatively more abundant, possibly revealing the stress-induced activation of a limited pool of progenitors.

The Shannon diversity index was used to assess the overall clonal repertoire composition of in vivo–derived BM $CD34^+$ and myeloid cells. In the first months after treatment, clonal diversity was lower then measured in the in vitro–transduced cell population but later increased and stabilized at 12 to 18 months (Fig. 3C). As predicted by the slower dynamics of thymic reconstitution, T cells showed different kinetics with increasing diversity from 3 to 6 months and stabilization to levels similar to other lineages at 9 to 18 months after gene therapy.

We next compared ISs retrieved from BM $CD34^+$ cells, myeloid, and lymphoid lineages in each patient, applying stringent analytical filters to take into account sample impurity and cross-contaminations. This analysis unveiled a group of ISs that was shared among these data sets (Fig. 4A). The shared ISs in $CD34^+$ cells and myeloid/lymphoid lineages increased from 18.2 to 27.2% at 1 year after gene therapy in Pt1. A higher proportion of integrations shared with other lineages (44.1%) was detected in BM CFC from Pt1. A substantial fraction of the ISs retrieved from BM $CD34^+$ cells or CFC at 1 year after gene therapy (Fig. 4B) could be traced back to earlier follow-up samples of mature cells, pointing to the efficient engraftment of self-renewing gene-marked HSPCs.

Our data allowed an estimation of the number of transduced HSPCs contributing to hematopoiesis in vivo. Vector integrations were analyzed in two independent samplings (12 and 18 months after gene therapy) of purified PB myeloid cells, which are short-lived and provide a readout of stem cell output. Using the Petersen/Schnabel model estimator of population size (*44*), we calculated a theoretical minimal number of about 6300 and 1700 transduced active stem cells in Pt1 and Pt2, respectively (table S25), which corresponds to about 1.5 to 2.5 in 1×10^5 infused $CD34^+$ cells.

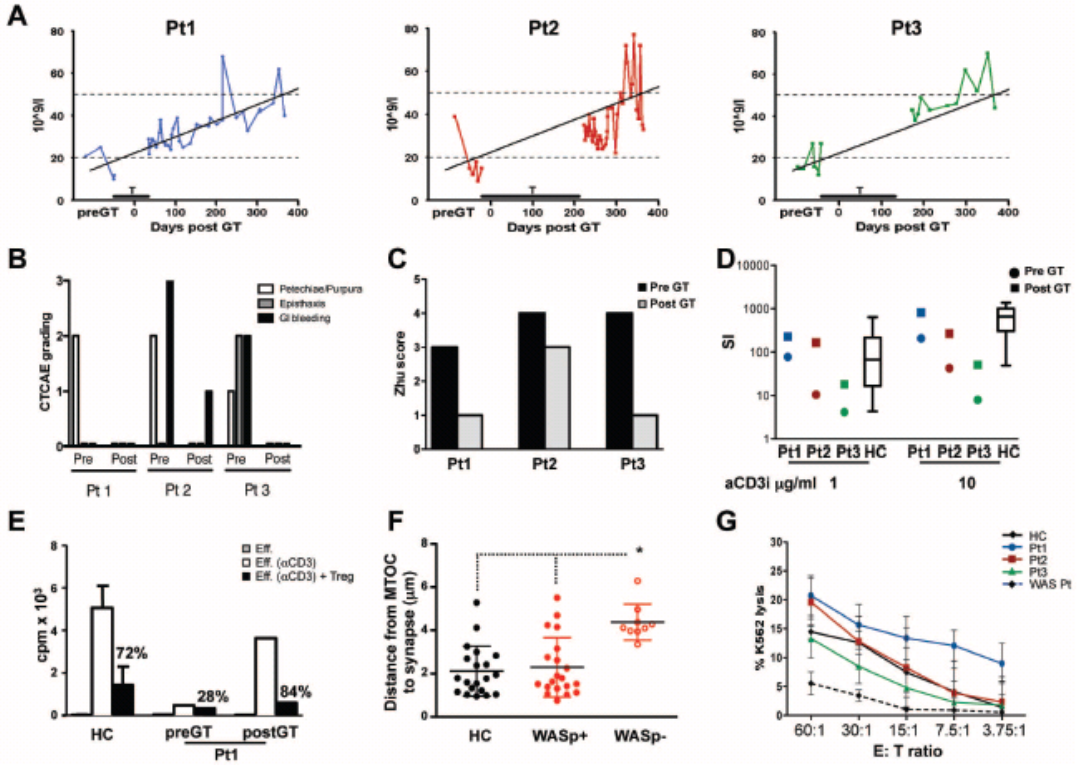


Fig. 2. Clinical features and immune function of WAS patients after gene therapy. (**A**) Platelet counts before and 1 year after gene therapy. Kinetics of platelet counts during the first year of follow-up, analyzed by a mixed linear model applied to the individual repeated counts. Platelet transfusions (T) are indicated by a horizontal bar. (**B**) Summary of bleeding events. Each of the three categories [skin manifestations (petechiae/purpura), epistaxis, and GI bleeding] was given a maximum grading, according to Common Terminology Criteria for Adverse Events (CTCAE) (v3.0). Patients were evaluated at 1 year after gene therapy following discontinuation of platelet transfusions (see Materials and Methods). (**C**) Disease score (Zhu score) evaluated pre- and 1 year post–gene therapy (*39*). (**D**) TCR-driven proliferation in PB mononuclear cells from WAS patients (before and 1 year after gene therapy) or healthy controls (HCs). (Boxplot) Mean, first, and fourth quartile, 5th and 95th percentile; $n = 15$ subjects. SI, stimulation index. (**E**) T_{reg}-mediated suppression of effector T cells pre–gene therapy and 1 year post–gene therapy in Pt1, in comparison with healthy controls (mean ± SD, $n = 9$ subjects). Eff, unstimulated effector cells. Eff.(a-CD3), effector cells stimulated with allogeneic accessory cells and soluble mAb to CD3. T_{reg} cells (sorted for $CD4^+/CD25^{high}/CD127^{low/neg}$ cells), were added to effector cells at a 1:1 ratio, and proliferation was measured by ^{3}H-thymidine incorporation. Percentages indicate inhibition of proliferation. (**F**) Formation of NK immunological synapse. Distance of the microtubule organizing center (MTOC) from the immunological synapses in NK cells contacting K562 target cells. Analyses were performed by confocal microscopy in Pt1 18 months after gene therapy. Patient NK cells were identified as positive or negative for WASP expression by antibody to WASP. (**G**) NK cell cytotoxic activity. Shown is the percentage of lysis of K562 human erythroleukemia cells by patient-derived PB mononuclear cells, as measured in a chromium-release assay.

Comparison of Lentiviral Versus γ-Retroviral "Integrome" in Gene Therapy of WAS

We compared the integration profile of LV-w1.6W vector with 11,294 unique ISs retrieved from two WAS patients enrolled in the γ-retroviral gene therapy trial (*12*) collected over a similar time frame and analyzed through the same mapping pipeline to obtain a comparable data set (table S10). The genomic insertional pattern of LV-w1.6W vector was similar in all three patients and fit the classical distribution of lentivirus showing a strong tendency to integrate within transcriptional units (fig. S15B) and cluster in gene-rich regions (Fig. 5A and fig. S15, A and B). No major difference was observed in the genomic distribution of LV-w1.6W

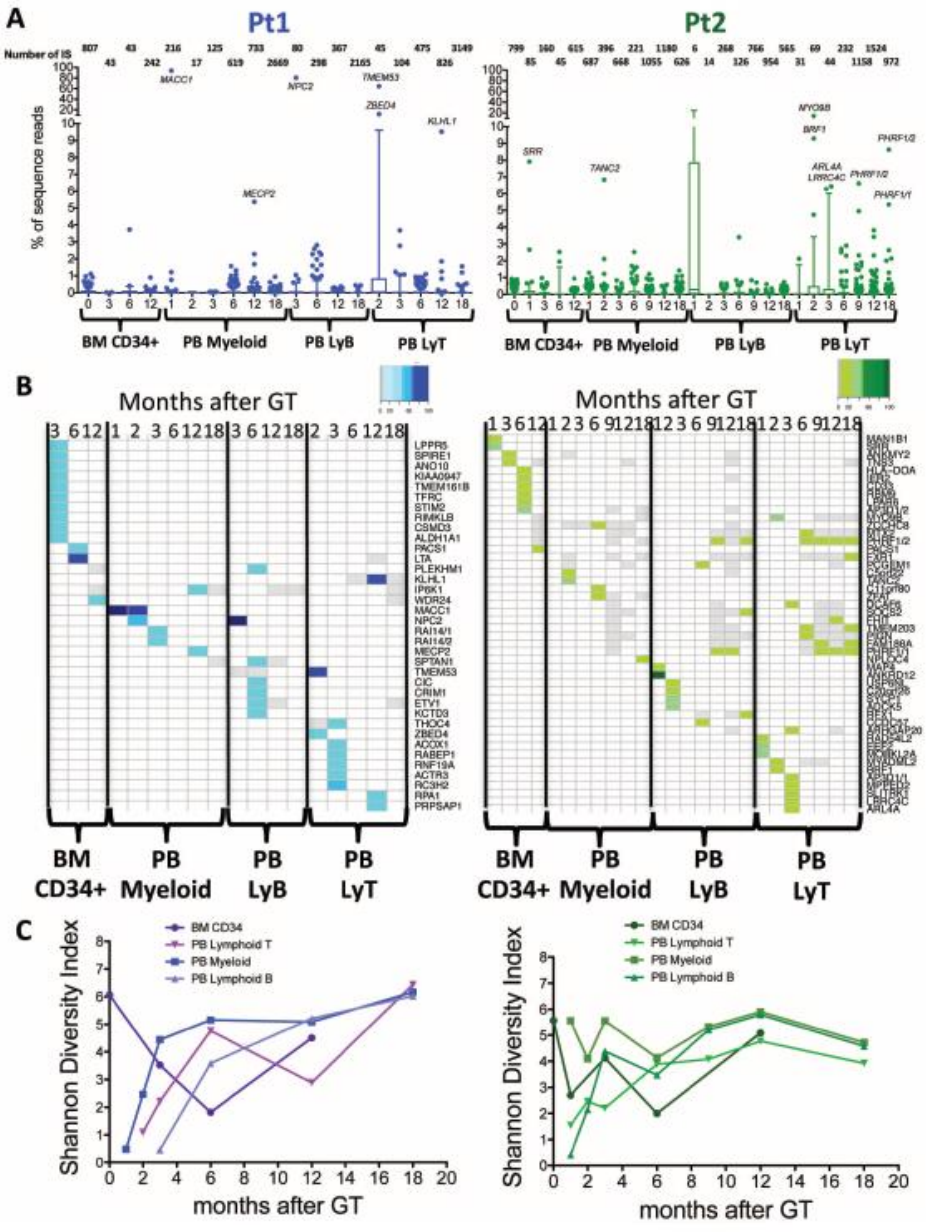


Fig. 3. Long-term polyclonal engraftment of gene-corrected HSPC, assessed by longitudinal integration site profiling. (**A**) Distribution of sequence reads in different lineages at different time points. Each boxplot shows the distribution of sequence reads in two WAS patients in the different lineages. Percentages of sequence reads for each IS are calculated over the total number of sequence reads from the same source (BM or PB) and time point. The *x* axis indicates months after gene therapy; the *y* axis shows percentage of sequence reads. IS outliers (over 95th percentile of IS data set from same time point) are shown as dots. The total number of ISs is shown on top of each boxplot for the relative lineage and time point. Genes proximal to IS representing more than 5% of sequence reads from the same source and time point are reported on top of outlier dots. (**B**) Top-contributing ISs inside each lineage in BM CD34$^+$ cells, PB myeloid, B and T cells in two WAS patients. Percentages of sequence reads for each IS are calculated over the total number of sequence reads from the same lineage. Top IS shown in the heat maps account for more than 5% of sequence reads from their lineage. Each column corresponds to a time point (months after gene therapy), and each row to a given top IS based on sequence reads. Color intensity on the heat maps represents for each IS the percentages of relative sequence reads on total sequence reads from the same lineage and time point, ranging from white (IS not detected at that time point) to gray (IS detected at percentages lower than 5% of sequence reads from that time point) to gradient of colors (IS detected at more than 5% of sequence reads from that time point). (C) Diversity of ISs in different lineages and time points. For each IS data set from the same lineages and time points of (A) and (B), a Shannon diversity index was calculated and plotted.

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in the in vitro– versus patients-derived samples (fig. S16A). By contrast, the profile found in the cells from patients treated with γ-retroviral gene therapy was very different, showing a skewed profile toward transcription start sites (fig. S15A).

As reported in previous studies (*45*, *46*), vector integration site selection could be influenced by the chromatin status of target cells during transduction and infection. Thus, we analyzed the density distribution of histone modifications mapped in $CD34^+$ cells (*47*) on a 100-kb window surrounding the ISs from the two cohorts of patients (Fig. 5B and fig. S20). The probability density map showed that certain histone modifications were equally preferred (H3K4me1) or avoided (H3K9me3 and H3K27me3) by lentivirus and retrovirus integrations. In addition, no major differences were observed between in vitro and in vivo lentivirus data sets. By contrast, other chromatin features—such as H3K4me3, H3K36me3, and H2AZ, marking, respectively, transcription start sites, coding, and enhancer/promoter regions of actively expressed genes—were present at differential intensity around ISs of the two vectors (Fig. 5B). Of note, the concomitant low density of H3K4me3 and H2AZ seems to provide an optimal chromatin environment for lentivirus insertions (figs. S17 to S19) and help explain the different genomic distribution observed for retroviruses.

We also analyzed the ontological categories of the genes targeted by vector integration sites in the data sets from the two patient cohorts by using the GREAT (Genomic Regions Enrichment of Annotations Tool) software (*48*) (Fig. 5C). A broad spectrum of biological categories, including antigen presentation, response to virus, chromatin modification, and gene silencing, was represented among the lentivirus targeted genes. In contrast, the retroviral insertions were more often proximal to genes related to hematopoietic system maintenance, as well as to hematological diseases. Of note, the vast majority of gene categories targeted by ISs in our LV-w1.6W patients were also targeted in the previous ALD gene therapy trial (*27*) (figs. S21

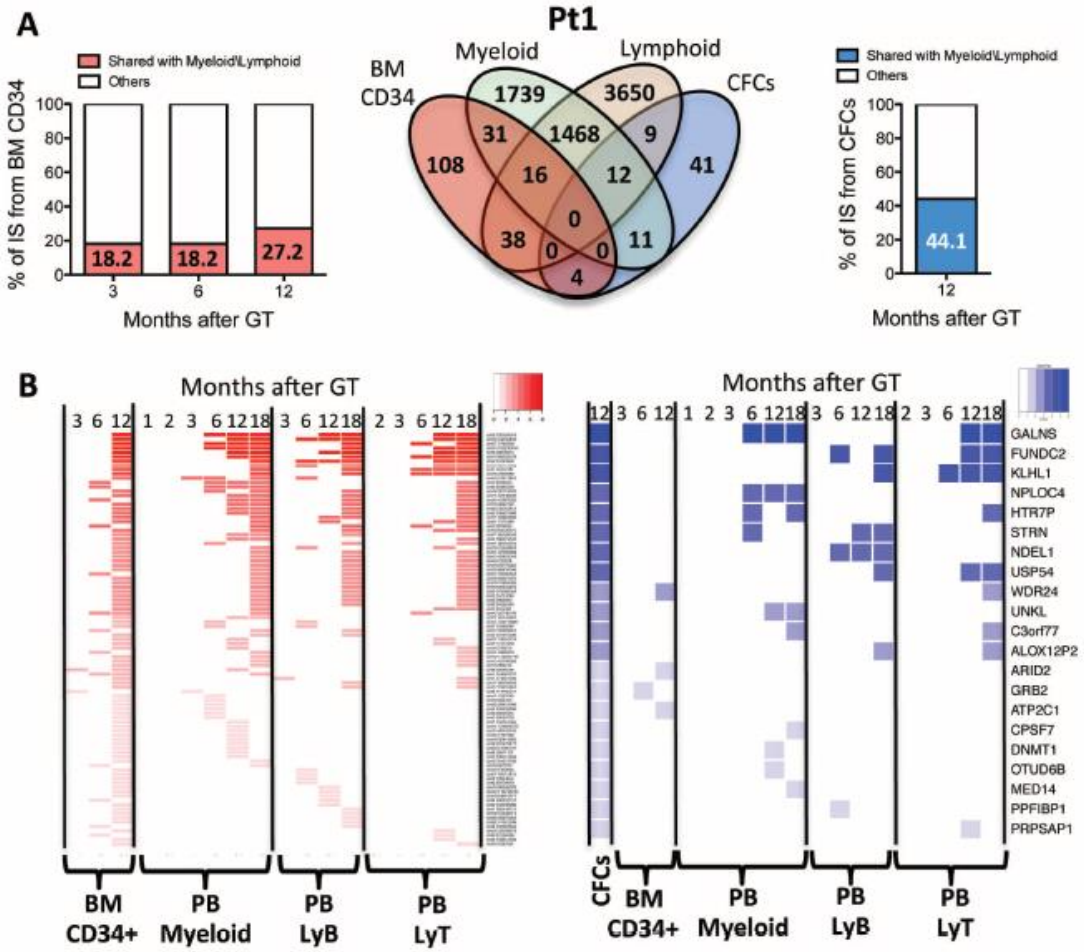

Fig. 4. Multilineage engraftment and activity of gene-corrected HSPC. (**A**) Multilineage detection of identical IS. Venn diagram show overlaps among $CD34^+$, myeloid, lymphoid cells, and CFC IS data sets from Pt1. Column graphs show percentage of $CD34^+$ cells and CFC IS from Pt1 shared with myeloid\lymphoid lineages (red and blue portion of column, respectively) at different months after gene therapy. (**B**) Detection of shared ISs over time. Heat maps show $CD34^+$ cells and CFC IS at different time points shared with the four lineages of Fig. 3. Each column shows a lineage and a time point and each row a shared IS belonging to $CD34^+$ cells (red) or CFC (blue). The intensity of colors indicates degree of IS detection in multiple lineages and time points from highly shared ISs (high intensity of red and blue) to ISs shared with a single lineage and time point (light red or blue).

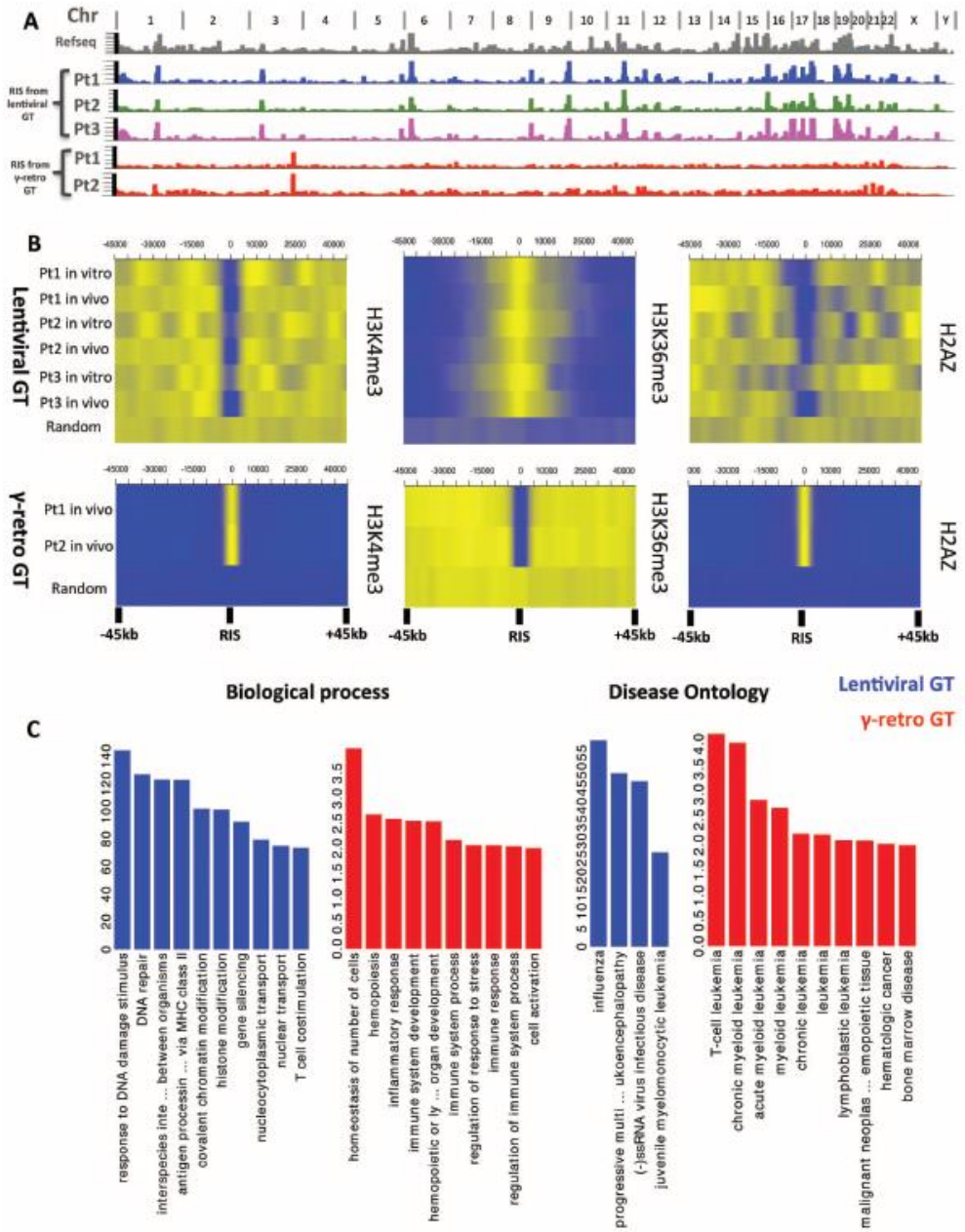

Fig. 5. Comparative analysis of vector integration sites in patients treated by gene therapy with lentiviral or γ-retroviral vectors. (A) Genomic distribution of ISs from five patients with WAS, treated by lentiviral ($n = 3$ subjects) or γ-retroviral ($n = 2$ subjects) vectors. Chromosome numbers are reported at the top of the graph. Refseq genes and IS frequency distributions are shown in bins of 1 million base pairs (Mbp) (gray, colored/red columns, respectively). (B) Chromatin modifications surrounding ISs. Probability density distributions of histone modifications mapped on $CD34^+$ cells in a ±45-kb window surrounding IS are shown as heat maps. The color intensity shows under-representation (blue) or over-representation (yellow) of each histone modification as compared with a random in silico–generated reference. Each row represents an in vitro or in vivo IS data set from the patients treated with lentiviral or γ-retroviral vectors. (C) Gene ontology of genes proximal to ISs. Biological process and diseases significantly associated with the functions of genes proximal to ISs in lentiviral and γ-retro gene therapy are represented as blue and red bars, respectively. The values on the *y* axis show the fold enrichment scale for gene ontology categories.

and S22). Thus, differently from retroviruses, LV-w1.6W has a lower probability to interfere with the expression of genes involved in transformation of hematopoietic cells due to its wider genomic distribution.

We next assessed the presence of common insertion sites (CIS) to study potential insertional hotspots at the time of transduction and define surrogate markers of in vivo selection of clones. CIS were detected using a canonical statistical method that assumes a random distribution of vector integrations (*49*) and a novel analysis for CIS discovery based on a genome-wide Grubbs test for outliers (for details on both methods, see supplementary text). The highest-ranking-order WAS lentiviral CIS were *KDM2A* (targeted by 125 integrations), *PACS1* (117) and *TNRC6C* (94) (table S23), which are well known hot spots in preclinical studies of lentiviral vectors (*50*, *51*) and in the ALD lentiviral gene therapy trial (*27*) and have not been associated with clonal

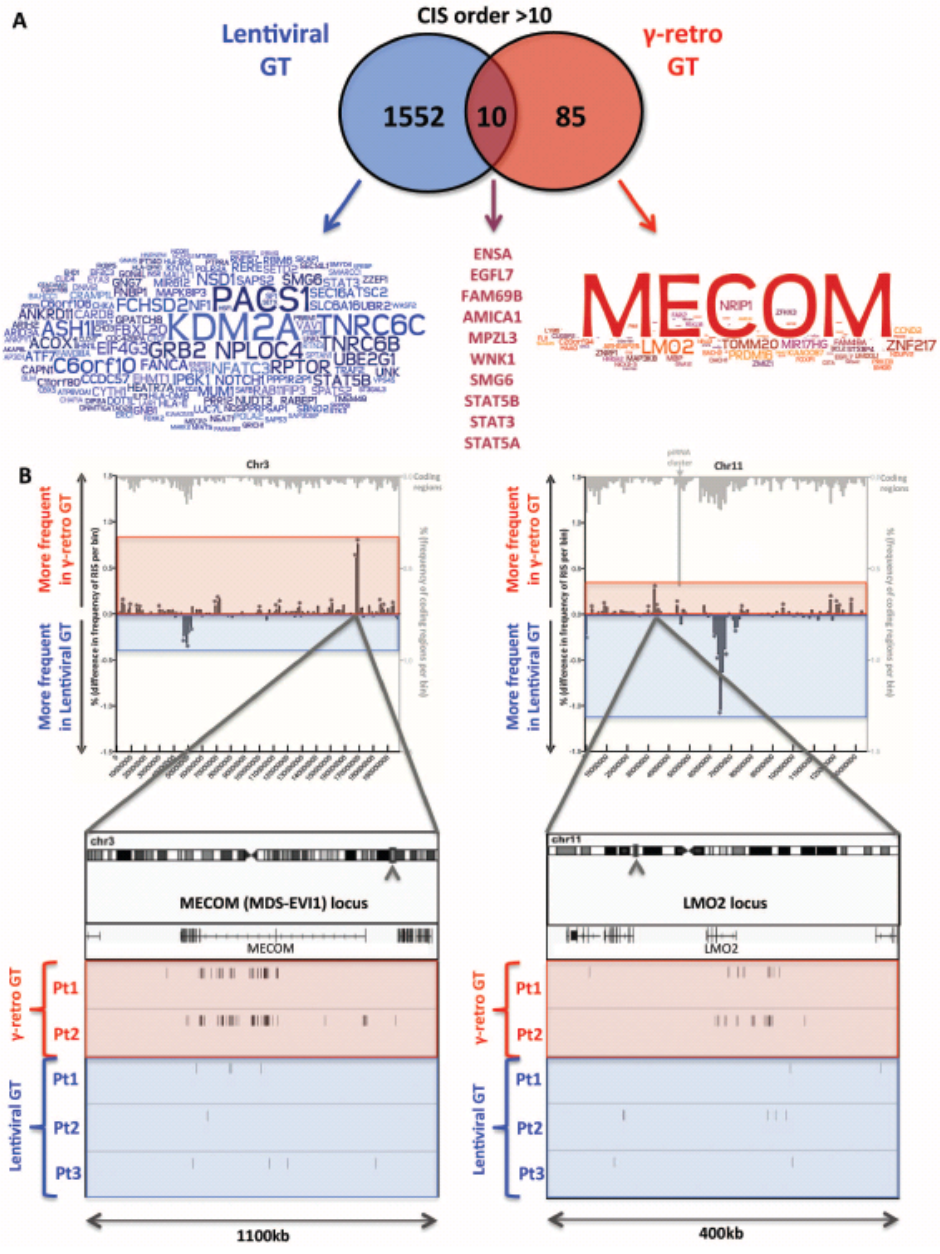

Fig. 6. Common insertion sites and oncogenic hits in lentiviral versus γ-retroviral gene therapy. (**A**) Overlaps among CIS genes in lentiviral and γ-retroviral gene therapy. Genes proximal to CIS of order >10 are reported in the overlapping circles for lentiviral and γ-retro gene therapy (blue and red, respectively). Word clouds show the intensity of ISs clustering in each of the CIS genes (the bigger the gene name, the higher the number of ISs inside or in the proximity of that gene). The names of the 10 CIS genes detected in both gene therapy trials are reported on the right at the intersection between the circles. (**B**) Incidence of ISs at two oncogenes in patients treated with lentiviral or γ-retroviral gene therapy. The comparative frequency distributions of insertions from lentiviral and γ-retroviral gene therapy in bins of 2 kb are shown for chromosomes 3 (left panel) and 11 (right panel). Mirror graph shows, for each bin, more frequent insertions in γ-retroviral gene therapy (columns above the *x* axis) and more frequent insertions in lentiviral gene therapy (columns below the *x* axis) (*$P < 0.05$, Fisher exact test). Light gray columns at the top show transcriptional unit frequency for the same bins. Boxes at the bottom show a detail of *MECOM* and *LMO2* loci, where ISs from the three lentiviral and two retroviral gene therapy patients are shown as sticks below the gene transcript. Raw IS data from the patients treated with the γ-retroviral vector were generated by Boztug *et al*. (*12*) and reprocessed through our informatic pipeline (table S3).

expansion (Fig. 6A and fig. S40). In contrast, γ-retroviral vector was found clustered in vivo in the *MECOM* (*MDS-EVI1*) and *LMO2* loci (Fig. 6A), hosting 67 and 15 of 11,294 integrations, respectively (*12*). In the current LV-w1.6W gene therapy trial, these two genes were hit only by 10 and 5 out of 33,363 integrations, respectively (Fig. 6B). Additionally, the relative sequence reads associated to these ISs (0.44% and 0.05% of reads from the same time point and sample source for *MECOM* and *LMO2*, respectively) were below the average of all other insertions, and these specific integrants were not consistently detected over time. These observations were confirmed for insertions near other genes previously involved in clonal dominance, such as *CCND2* and *PRDM16* (fig. S15C). The *HMGA2* locus was hit in both data sets (fig. S15C) but, in contrast to what was reported in the β-thalassemia patient (*28*), we did not observe over-representation of these integrations during follow-up. Together, these results indicate the lack of evident clonal expansions associated with CIS in proximity of known proto-oncogenes, suggesting that the insertional hot spots found in our trial are the product of vector integration biases at the time of transduction rather than in vivo genetic selection of ISs conferring a selective growth advantage.

Discussion

We have shown that infusion of autologous HSPC transduced with a lentiviral vector encoding a functional WASP gene resulted in robust and stable multilineage engraftment in three WAS patients who had been pretreated with a reduced intensity preparative regimen. The gene-corrected blood cells expressed WASP under control of a reconstituted WAS promoter. The patients showed improved immune function and amelioration of clinical manifestations of the disease, including protection from bleeding and severe infections, as well as resolution of eczema.

In this trial, the in vivo gene marking of hematopoiesis was superior (~25 to 50% of BM progenitors and mature PB myeloid cells) to that described in other lentiviral-based gene therapy trials for ALD and β-thalassemia (*27*, *28*), which used myeloablative preparative regimens. The use of highly purified and homogenous batches of lentiviral vectors, optimized culture conditions and vector exposure (*34*), and the infusion of cells shortly after transduction likely accounts for the robust engraftment. In a parallel trial using the same procedures to treat patients with metachromatic leukodystrophy (MLD) (*52*), in vivo gene marking of HSPC was even higher (45 to 80%). The preparative conditioning regimen in the latter trial was more intensive, suggesting that chemotherapy regimens can be modulated based on the engraftment threshold required for therapeutic benefit. Notably, stable engraftment of marked progenitor cells in vivo was higher in this lentiviral vector trial than in the previous WAS trial based on γ-retrovirus vector (3 to 22%) (*12*). A selective advantage of lymphoid cells carrying a functional WAS gene (*12*) was observed in both clinical trials, in line with previous studies in the murine disease model (*12*, *30*, *33*, *53*).

Although gene therapy did not normalize platelet counts in the patients, the counts did increase significantly and the platelets were of normal size, improvements that were sufficient to protect patients from bleeding and related disorders. Considering that the BM must contain a mixed population of WASP-positive and WASP-negative megakaryocytes and only platelets with normal levels of WASP were detected in peripheral blood, it is conceivable that a threshold level of WASP is required for platelets to exit the BM and/or to survive in the periphery. It is noteworthy that platelet counts in WAS patients treated by lentiviral gene therapy are in the range measured in patients with mixed donor/host chimerism after successful allogeneic BM transplant (*7*). A more intense conditioning regimen or the use of a lentiviral vector with a stronger promoter might result in a greater number of gene-corrected platelets. However, these options should be carefully balanced with the higher risk of toxicity, as reported for the conditioning used in allogeneic BM transplant, as well as of transactivation and insertional oncogenesis associated with strong enhancers and promoters, such as those of γ-retroviral vectors (*54*).

A consistent multilineage recapture of ISs 12 months after gene therapy indicated the active contribution of gene-corrected long-term HSPC to hematopoiesis. Our results lead us to propose that a takeover of hematopoietic output by long-term HSPC occurs at a defined time window after gene therapy, generating a diverse clonal repertoire in the blood progeny. The high proportion of ISs found in clonogenic BM progenitors that are shared with mature lineages validates IS-based tracking of stem cell dynamics and the colony assay as a representative surrogate readouts of in vivo HSPC activity. The calculated minimal number of 1700 to 6300 transduced active stem cells engrafted in vivo is consistent with data from the parallel MLD trial (*52*) and with estimates that repopulating HSPCs account for 1 out of 10^4 to 10^5 infused $CD34^+$ cell populations (*55*), considering the impact of cell manipulation and the potentially limited BM homing after transplant.

The study of ISs from WAS patients treated with lentiviral or retroviral vector (*12*) allowed the first comparative study of the safety and clinical performance of two different vectors in the context of the same disease background. Our analyses expand previous observations that chromatin conformation and histone modifications differently affect the genomic distribution of retroviral vectors (*45*, *56*), with the identification of H3K4me3 and H2AZ as two major chromatin determinants of differential insertions between lentiviruses and retroviruses. Differently from the retroviral gene therapy trial, where clonal enrichment of ISs targeting oncogenes like *MECOM*, *CCND2*, and *LMO2* had already occurred at early follow-up, oncogenic ISs were not overrepresented in our patients, in agreement with previous preclinical and clinical studies (*27*, *34*, *51*). Many genes and related categories targeted by lentivirus in our WAS patients were also hit in the parallel MLD trial (*52*) and in a previous ALD lentiviral trial (*27*), where no clonal dominance or leukemia have been reported up to 5 years after treatment (*14*). These findings are particularly relevant considering the high risk of lymphomagenesis described in WAS patients. The overall tendency of LV-w1.6W to distribute over megabase-wide areas and a broader spectrum of gene classes, together with the lower transformation potential of our SIN LTR-based construct, likely make lentiviral vector less prone to aberrantly interact with proto-oncogenes and induce genotoxicity. Collectively, although a definitive conclusion on safety must await the long-term observations, these data suggest that lentiviral-based HSPC gene therapy is safer than retroviral gene therapy.

Materials and Methods

Clinical Protocol and Patients

A phase I/II clinical trial of HSPC gene therapy for WAS was initiated in April 2010, after authorization by Istituto Superiore di Sanità on 15 March 2010 and by San Raffaele Scientific Institute Ethics Committee on 2 April 2010 (Eudract no. 2009-017346-32). The study promoter is HSR-TIGET, San Raffaele Hospital, Italy, and the financial sponsor of the study is Telethon Foundation. The medicinal product received Orphan Drug Designation (ODD) by the European Medicines Agency (EMA) (EU/3/12/998) and the FDA (ODD#10-3043).

Male children with WAS suffering from a severe clinical condition (Zhu clinical score ≥ 3) or severe mutation/absent WASP expression, without a suitable matched donor for allogeneic transplant or ineligible for HSPC transplantation (because of age >5 years or clinical features), were eligible for the study. The parents of all subjects provided written informed consent for experimental treatment. Details on the clinical study can be found at ClinicalTrials.gov (no. NCT01515462). Biological samples were obtained from WAS patients, healthy children or adults, with approval of the San Raffaele Scientific Institute's Ethics Committee and consent from parents or subjects.

Lentiviral Vector

The lentiviral vector used in this clinical trial is a third-generation SIN vector derived from HIV, named pCCLsin.cPPT.hw1.6.hWAS.WPREmut (abbreviated as LV-w1.6W), and it has been previously described (*3*, *34*). This is a pseudotyped vector made by a core of HIV-1 structural proteins and enzymes, the envelope of the vesicular stomatitis virus (VSV), and a genome containing HIV-1 cis-acting sequences, no viral genes, and one expression cassette for the WAS transgene.

In the cassette, the endogenous 1.6-kb promoter of WAS controls the expression of WAS cDNA. The three vector components (core, envelope, and genome) are transiently expressed in vector producer cells by four different constructs: two core packaging constructs, the envelope construct, and the transfer vector construct. Only the vector construct is transferred and integrated into the target cells.

Clinical lots of vectors were produced under Good Manufacturing Practice (GMP) conditions by MolMed S.p.A. (Milan, Italy), a certified GMP facility. Vectors were produced by large-scale process based on transient quadritransfection in 293T cells, followed by purification through endonuclease treatment, anion exchange chromatography with gradient elution, gel filtration, resuspension in serum free media, 0.22 μm filtration, and aseptic filling (fig. S1). Each lot was characterized in terms of titer, potency, purity, and safety aspects (tables S1 and S2).

CD34⁺ Cell Gene Transfer

Patients' $CD34^+$ cell manipulation and transduction were performed at MolMed S.p.A. as detailed in fig. S2. Patients' BM was diluted, stratified with Lymphoprep (Axis Shield, Oslo, Norway), and centrifuged in order to collect mononuclear cells (MNC). $CD34^+$ cell-positive selection from BM MNC was performed using immunomagnetic beads (CliniMACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) and an immunomagnetic enrichment device. For MPB leukapheresis, $CD34^+$ cells were processed using the CliniMACS device. Two independent lots of transduced cells were prepared for Pt1 (one for BM and one for MPB). The number of $CD34^+$ cells collected from the BM and used for transduction ranged from 4.4 to 14.5×10^6/kg (table S3). MPB-derived $CD34^+$ cells in Pt1 were 6.5×10^6/kg after thawing on day 3 (table S3). Purified $CD34^+$ cells were seeded on VueLife bags (American Fluoroseal Corp., Gaithersburg, MD, USA) at 1×10^6 cells/ml in serum-free CellGro SCGM Medium (Cell Genix Technologies, Freiburg, Germany) in the presence of cell culture grade stem cell factor (SCF) 300 ng/ml (Amgen Inc., Thousand Oaks, CA, USA), FLT3-L 300 ng/ml, thrombopoietin (TPO) 100 ng/ml, and IL-3 60 ng/ml (all from Cell Genix Technologies). After 24 hours of prestimulation, cells were transduced with LV-w1.6W at 100 multiplicity of infection (MOI) for two hits of transduction with a wash period of 12 to 14 hours between the vector exposures. Transduced cells were tested for immune phenotype, sterility, endotoxin, mycoplasma, large T antigen, E1A DNA, large T antigen DNA, clonogenic content, transduction efficiency, and replication-competent lentivirus (table S4). Cells were resuspended in saline and transferred in a syringe for infusion. An aliquot of cells was cultured in Iscove's modified Dulbecco's medium (IMDM), 10% fetal bovine serum (Cambrex, East Rutherford, NJ, USA) with the same cytokines at 20 ng/ml concentration and harvested after 15 days to perform proviral integration evaluation by qPCR, WASP expression measurement, and integration profile analysis.

Rationale for Conditioning Regimen

Allogeneic HSPC transplant for WAS patients usually requires a high-dose myeloablative and immune suppressive regimen to deplete host bone marrow stem cells and prevent rejection or graft-versus-host disease. Previous studies in the context of ADA-SCID gene therapy indicated that a reduced-dose chemotherapy regimen with busulfan at 25% of standard dose was sufficient to achieve substantial engraftment of gene-corrected cells while reducing conditioning-related toxicity (*9*, *15*, *16*). Because WAS requires higher levels of stem cell correction with respect to SCID, our chemotherapy regimen is based on administration of both busulfan and fludarabine as depleting agents for endogenous HSPC. The dose of busulfan and fludarabine are ~50% and 30% of the ones employed in standard allogeneic transplantation, respectively. Fludarabine was also used to break the homeostasis in the compartment of early lymphoid progenitors and to favor the establishment of a pool of corrected naïve T cells in the periphery, because the selective advantage for $WASP^+$ cells is thought to take place mainly through peripheral expansion rather than during thymic differentiation (*57*, *58*). mAb to CD20 was introduced as a depleting agent for B cells, and particularly of autoreactive cells, thereby facilitating the engraftment and expansion of gene-corrected B cells expressing WAS. In addition, antibody to CD20 was used as pre-emptive treatment for lymphoproliferative disorder due to Epstein-Barr virus (EBV), which represents a high risk factor for the development of lymphoma in WAS patients.

Patients' Treatment

In Pt1, mobilization of peripheral blood stem cells after granulocyte colony-stimulating factor (G-CSF) stimulation was performed to collect back-up HSPCs and store an adequate HSPC dose for subsequent transduction and reinfusion in case of need. In Pt2 and Pt3, due to their younger age, only a BM back-up was performed. A central venous catheter was implanted in all patients.

On day 3, autologous BM was collected from iliac crests under general anesthesia. A reduced-intensity conditioning regimen was then administered before reinfusion of the autologous-engineered HSPC. It consisted of i.v. busulfan [bodyweight-based and area under the curve (AUC) adjusted-dose; range, 0.8 to 1.2 mg/kg/dose] administered consecutively in eight doses every 6 hours from days –3 to day –1 and i.v. fludarabine (30 mg/sqm/day) on days –3 and –2. The actual doses and AUC of busulfan received by each patient are summarized in table S5. A single dose of mAb to CD20 (Rituximab, 375 mg/sqm) was administered on day –22. Transduced autologous LV-w1.6W $CD34^+$ cells, manufactured as indicated above, were infused i.v. in 20 min. Patients received antibacterial, antifungal, anti–Pneumocistis jirovecii, and antiviral prophylaxes according to local standards. The treatment was administered at the Pediatric Immunology and BM Transplantation Unit, and patients were hospitalized in isolation for 40, 70, and 43 days, respectively.

Patients' Features and Clinical Course After Gene Therapy.

The clinical features before treatment and the current clinical conditions of the first three patients treated are summarized in Table 1. Pt1 was a 5.7-year-old boy lacking an human lymphocyte antigen–compatible donor, with clinical history of recurrent, frequent ENT and viral infections, skin petechiae, and moderate-severe eczema (Zhu score 3). His clinical course after treatment was uneventful. The second and third patients (Pt2 and Pt3) were younger and had a Zhu score of 4, due to history of severe infections, severe eczema, and GI bleeding. Pt2 was affected by a colitis associated with HHV-6 and CMV infection, as well as persistently elevated inflammatory indexes and vasculitis-like skin manifestations. Pt3 had important feeding problems, with severe GE reflux and food aversion, and was fed by nasogastric tube. Pt2 developed an autoimmune thrombocytopenia, which is frequently observed after allogeneic transplant and was treated with IVIG and mAb to CD20. The patient experienced a Gram-negative sepsis complicated by a disseminated intravascular coagulation and acute respiratory distress syndrome (ARDS), for which he received steroids for about 5.5 months. Pt3's clinical course was characterized by respiratory infections related to aspiration due to the underlying GE reflux, including an episode of interstitial aspiration pneumonia, complicated by ARDS, and requiring steroid treatment for about 2 months. Both patients recovered well from these events without sequelae. No immune suppressive treatment was administered to the patients subsequently. Pt1 responded to vaccination with recombinant antigens, with production of specific antibodies and in vitro proliferative responses to tetanus toxoid.

Laboratory Studies

CFC Assay

CFC assay was performed, immediately after transduction or on ex vivo BM samples according to the manufacturer's procedure in Methocult medium (Stem Cell Technologies, Vancouver, Canada). At day 14, colonies were scored to determine number and type of colonies, singly picked and analyzed by qPCR to evaluate the percentage of transduction.

Flow-Cytometric Analysis

Surface staining of transduced $CD34^+$ cells was performed with mAb to CD34 (8G12) and mAb to CD45 (HI30) (BD Biosciences, San Jose, CA). Cells from PB of patients and healthy donors, purified by standard density gradient technique (Lymphoprep), were stained for the expression of surface markers CD3 (SK7), CD4 (SK3), CD8

(SK1), CD19 (SJ25C1), CD14 (MΦP9), CD56 (NCAM16.2), CD25 (2A3), and CD41 (HIP8) (BD Biosciences) and of intracytoplasmatic expression of WASP. Detection of WASP was performed after permeabilization (Cytofix/Cytoperm kit, BD Biosciences) by two lots of rabbit polyclonal antibodies to human WASP (one of which was a gift of H. Ochs) and followed by staining with a secondary Alexa 488 or 647 conjugated goat antibody to rabbit immunoglobulins (Ig) anti-rabbit antibodies. Tertiary rabbit antibodies to goat Ig (conjugated to Alexa 488 or 647) (all from Invitrogen, Carlsbad, CA, USA) were used. For analyses of TCR repertoire, 24 different TCR Vβ specificities were analyzed by flow cytometric analysis by IOTest Beta Mark kit (Immunotech, Marseilles, France) according to the manufacturer's procedure. Cells were acquired using a FACSCantoII (BD Biosciences) and analyzed with Flow Jo Software (Tree Star Inc., Ashland, OR, USA).

Western Blot Analysis

Western blot was performed as previously described (*3*, *34*), using antibody to human WASP (H250; Santa Cruz Biotechnologies, Santa Cruz, California, USA) and antibody to human glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, California, USA), followed by secondary horseradish peroxidase–coupled antibodies (DAKO A/S, Glostrup, Denmark).

Purification of Peripheral Blood and Bone Marrow Lineages

CD3, CD4, CD8, CD14, CD15, CD19, and CD56 cells were purified from MNC from PB. CD3, CD15, CD19, CD34, CD56, CD61, and glycophorin were purified from BM-derived MNCs by positive selection with immunomagnetic beads according to the manufacturer's procedure (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Determination of Vector Copy Number by Real-Time PCR

To evaluate the number of lentiviral vector copies integrated per genome, a qPCR was performed using specific primer and probes for human telomerase and lentiviral vector, as described previously (*34*). A reference standard was obtained from serially diluted transduced human T cell lines carrying one copy of integrated lentiviral vector. Results of integrated vector copies were normalized for the number of evaluated genomes. As a negative control, samples of untransduced cells were used. All the reactions were performed according to the manufacturer's instructions and analyzed with an ABI PRISM 7900 sequence detection system (Applied Biosystem, Foster City, CA).

Vector Integration Analyses and Bioinformatics

These methods are described extensively in the Supplementary Materials.

In vitro Suppression Assays

Suppression assays were performed as previously described (*59*). Briefly, $CD4^+CD25^{high}CD127^{-/low}$ T_{reg} cells and $CD4^+CD25^-$ effector T cells were isolated from MNC by fluorescence-activated cell sorting. Cells from healthy subjects were used as control. A total of 15×10^3 $CD4^+CD25^-$ effector T cells were stimulated by CD3-depleted antigen-presenting cells and 1 μg/ml of soluble mAbs to CD3 (Orthoclone OKT3, Janssen-Cilag). Suppressive activity of nT_{reg} cells was assessed by co-culture of effector T cells with nT_{reg} cells at a 1:1 ratio. Proliferation was evaluated by 16 hours liquid scintillation counting of 3H-thymidine (Amersham Biosciences) incorporation after 96 hours of stimulation.

Analysis of the NK Cell Immunological Synapse

NK cell immunological synapses were analyzed as previously described (*60*). In brief, NK cells were cocultured with K562 target cells for 30 min to permit the formation of conjugates. F-actin and WASP accumulation, as well as perforin and MTOC polarization were measured to determine synapse maturity. The distance of the MTOC from the synapse was measured using the Velocity software package (Improvision-Perkin Elmer, Lexington, MA). Mean distances of the MTOC to the immunological synapse are a measure of synapse maturity and were compared by using the Mann-Whitney rank sum test.

Chromium-Release Cytotoxicity Assay

The ability of NK cells to lyse major histocompatibility complex devoid target cells was performed using a standard chromium-release cytotoxicity assay (*61*). Briefly, K562 target cells were labeled with 60 μCi of $Na_2(^{51}CrO_4)$ (Perkin Elmer Inc., Waltham, MA, USA) per 10^6 cells for 2 hours at 37°C and resuspended at used concentration. Effectors MNC were isolated and coincubated at serial diluted effectors to target (E:T) ratios of 60:1, 30:1, 15:1, 7.5:1, and 3.75:1 in duplicates in a 96-well v-bottom plate (Corning Costar, NY, USA) for 4 hours at 37°C in the atmosphere of 5% CO_2. Radioactivity was measured in the culture supernatants by Canberra Packard Cobra II Auto Gamma Counter (Canberra Packard, Canberra, Canada). Percentage of specific target cell lysis was calculated as follows: (experimental.cpm-spontaneous.cpm)/(maximal.cpm-spontaneous.cpm) × 100. For spontaneous release, targets were cultured with medium alone, and for maximum release in 3% (v/v) Triton X-100 (Sigma, St. Louis, MO) in phosphate-buffered saline.

T Cell Response to Antibody to CD3

T cell response to antibody to CD3 was performed as previously described (*62*). Briefly, 0.1×10^6 PB MNC were seeded into a round-bottom 96-well plate in IMDM medium containing 5% human AB serum (Lonza, Basel, Switzerland) in the presence of coated 1 or 10 μg/ml of antibody to CD3 (Orthoclone OKT3, Janssen-Cilag). Proliferation was assessed by 3H-thymidine (Amersham Biosciences) incorporation after 72 or 96 hours of stimulation. Data were expressed as the maximal counts per minute (cpm) and stimulation index (SI, calculated as cpm/background cpm). For proliferation assay on untransformed T cell lines, overnight starved T cells were stained with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE), and 0.1×10^6 cells were cultured in a round-bottom 96-well plate coated with different concentration (from 0.01 to 10 μg/ml) of antibody to CD3. Proliferation was assessed after 72 hours evaluating CFSE dilution on a FACSCantoII, and data were analyzed with Flow Jo Software.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S46
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Chapter 4

"New approaches for the clinical management of children affected by hematological and immunological disorders undergoing bone marrow transplantation"

Ann Hematol
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ORIGINAL ARTICLE

Guidelines for the use of long-term central venous catheter in children with hemato-oncological disorders. On behalf of supportive therapy working group of Italian Association of Pediatric Hematology and Oncology (AIEOP)

F. Carraro · M. P. Cicalese · S. Cesaro · R. De Santis · G. Zanazzo · A. Tornesello · P. Giordano · A. Bergadano · M. Giacchino

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Abstract In the last 30 years, the use of long-term central venous catheters (CVC) is increased especially for children with hemato-oncological disorders. However, the use of CVC is associated to complications, as mechanical accidents, thrombosis, and infections that can determine a prolongation of hospital stay, an increase of costs, and sometimes life-threatening conditions that require urgent systemic treatment or CVC removal. CVC removal may be troublesome especially in neonates, infants, or any other "highly needed CVC patients"; in these selected cases, the prevention and treatment of CVC-related complications play a pivotal role and specific surveillance programs are crucial. While extensive literature is focused on CVC management in adults, no guidelines are available for children. To this aim, the first recommendations for the management of CVC infectious complication in pediatric age have been written after pediatric and adult literature review and collegial discussion among members of Supportive Therapy working group of Italian Association of Pediatric Hematology and Oncology. Compared to the adult age, the necessity of peripheral vein cultures for the diagnosis of CVC-related infection remains controversial in children because of the poorer venous asset and a conservative, pharmacologically focused management through CVC remains mandatory, with CVC removal to be performed only in selected cases.

Keywords Central venous catheter · Infections · Cancer · Pediatric age

Carraro F. and Cicalese M.P. equally contributed to this work.

F. Carraro · A. Bergadano · M. Giacchino (✉)
Pediatric Hematology Oncology and Bone Marrow Unit, Ospedale Infantile Regina Margherita, Turin, Italy
e-mail: mareva.giacchino@unito.it

M. P. Cicalese
Pediatric Immunology and Bone Marrow Transplantation Unit, IRCCS San Raffaele, Milan, Italy

M. P. Cicalese
San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), IRCCS San Raffaele, Milan, Italy

S. Cesaro
Pediatric Hematology Oncology, Azienda Ospedaliera Universitaria Integrata, Verona, Italy

R. De Santis
Pediatric Hematology Oncology Casa Sollievo della Sofferenza Hospital, CSS IRCCS, San Giovanni Rotondo, Italy

G. Zanazzo
Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy

A. Tornesello
Cattolica S Cuore University, Rome, Italy

P. Giordano
Biomedic Science and Human Oncology Department, University of Bari, Bari, Italy

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Introduction

Long-term central venous catheter (CVC) has been increasingly used in the last 30 years for the management of pediatric oncology patients. The CVC is fundamental to deliver safely multidrug chemotherapy and intensive support therapy such as antimicrobial agents, analgesics, blood products, hyperhydration, and hyperosmolar parenteral nutrition. CVC can be either tunneled (single lumen or multilumen) with a subcutaneous cuff adjacent to the catheter exit

site, i.e., Broviac Hickman or Groshong CVC, or totally implanted port system with subcutaneous reservoir [1, 2].

The use of CVC is associated to some adverse events, such as mechanical accidents, infections, and thrombosis that can determine a prolongation of hospital stay and an increase of costs. Moreover, in case of life-threatening situation, urgent removal of the CVC may be required. Catheter-related infections (CRI) cause considerable morbidity in patients with CVC; Gram+ bacteria, coagulase-negative staphylococci (CONS), and *Staphylococcus* aureus are the pathogens most frequently cultured, followed by *Candida* species [1].

The CRI incidence rates reported in literature are 1.7–11.3 cases per 1,000 CVC days [3–5]. In pediatric hemato-oncology patients with long-term CVC, the reported rates were 1.4 per 1,000 CVC days for implantable port (Port-a-Cath) and 1–4.6 per 1,000 CVC days for partially implanted CVC [4–6]. From 27 to 46 % of infected CVC are removed because of persistent bacteremia, despite an adequate systemic antibiotic treatment [4, 5]. Once infected, CVC removal may be difficult, especially in neonates, infant children, or any other patients who have a limited reserve of venous vessels for different reasons (so-called "highly needed CVC patients"). The prevention and treatment of CVC-related complications thus play a pivotal role, and specific surveillance programs are crucial both to monitor the risk factors for CVC complications and to improve their management [1, 4, 6].

While an extensive body of literature is focused on CVC in adults, no guidelines are available for the pediatric patients. To this aim, we reviewed the pediatric and adult literature to propose specific guidelines for the pediatric onco-hematological patients.

Methods

The Working Group (WG) of Supportive Therapy of Italian Association of Pediatric Hematology Oncology (AIEOP) promoted the definition of the guidelines for the management of long-term catheter (CVC) in pediatric onco-hematological patients in collaboration with the WGs of Infection, Coagulation, Surgery and Nursing. In a first meeting in 2011, each WG indicated members skilled in the arguments of CVC and infection. This expert panel defined the topics to discuss and performed a literature search. The key words used for the selection of the studies were "central venous catheter, bloodstream infections, catheter-related infections, oncologic patients and pediatric malignancy." The search was limited to English language papers and the period analyzed was from January 2000 to January 2012. Reference list of papers selected by literature was also used as a source for recommendations. The results of the search were discussed and scored by the members of the panel using the scoring system proposed by Infectious Diseases Society of America (Table 1). The recommendations were proposed and discussed in a second meeting among all members of WGs and the results of this debate were the basis for the final document. The definite document was approved by all members in a third meeting during 2012.

Results

Diagnosis The definitions of CRI according to the international guidelines [1] are summarized in Table 2. Surveillance blood cultures are not indicated in onco-hematological patients and in children who undergo hematopoietic stem cell transplantation [1, 7].

In febrile cancer patients with CVC, the signs and symptoms of infection can be shaded by neutropenia and, in leukemic patients, by concomitant steroid treatment [8]. Therefore, a febrile cancer patient requires an early diagnostic investigation including at least two sets of blood cultures, blood sampling being performed by aseptic technique [9]. In patients with CVC, most of experts recommend to obtain a blood culture set from peripheral vein simultaneously with blood culture set from each lumen of the CVC, before the initiation of an empirical antibiotic therapy (AII) [1, 2]. The amount of blood taken from CVC and from peripheral vein must be equal [1] (AII). Moreover, the use of blood cultures sets for aerobic and anaerobic bacteria is recommended in immunocompromised patients [1] (BII).

In pediatric patients, the compliance to this indication may be difficult in the routine practice, especially in neonates and infants or in children with poor venous asset or with hypotension and shock. On the other hand, CVC is largely used to sample the patients for routine blood tests to avoid the use of peripheral vein that may cause continuous distress and painful experience for patients and parents.

The major advantage of obtaining a set of blood culture from peripheral vein is the possibility to differentiate bacteremia from CRI, according to the points 1, 2 and 3 listed in Table 2. It has been demonstrated that the differential time to positivity (dTTP) is a sensitive and specific method to distinguish bacteremia from CRI. Using a dTTP cutoff of 120 min (or 150 min in pediatric patients) between blood culture from CVC and blood culture from peripheral, the sensitivity resulted in 91 % and the specificity in 94 % [2–4, 8, 10]. Therefore, this method is highly recommended to diagnose a CRI (AI). Moreover, in a patients with double-lumen CVC, a dTTP of 180 min between the blood cultures taken from different lumens allows to distinguish the infected lumen responsible of CRI [3, 11, 12] (AII). In centers that do not routinely adopt microbiological quantitative culture techniques or differential time to positivity method, this expert panel considers reasonable the choice to withdraw the blood culture only from CVC of a febrile pediatric patients (CII). Moreover, in a patient with single-lumen CVC, it is recommended to perform at least two

Table 1 IDSA-United States Public Health Service grading system for ranking recommendations

Quality of evidence		Strength of recommendation	
I	Evidence from ≥1 properly randomized, controlled trial	A	Good evidence to support a recommendation for use
II	Evidence from ≥1well-designed clinical trial, without randomization; from cohort or case-controlled analytic studies (preferably from >1 center); from multiple time series; or from dramatic results from uncontrolled experiments	B	Moderate evidence to support a recommendation for use
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees	C	Poor evidence to support a recommendation

blood cultures in a short span of time (15–30 min) before the start of antibiotic treatment to distinguish a real bacteremia/CRI from a false-positivity due to contamination of health personnel [1, 4, 10] (BIII). In fact, in case of isolation of common skin bacteria (CONS, *Corynebacteria* apart from *Campylobacter jejuni*, *Propionibacteria*), there is a general agreement that two blood cultures are needed to attribute a causative role to these opportunistic germs [1, 3, 8] (AI). The execution of at least two blood cultures at a short span of time increases the sensitivity of the test: 65 % with one blood culture, 88 % with two blood cultures [13].

In the presence of signs of CVC exit site or CVC tunnel infection, a skin swab must be performed [1–3, 8] (AI). In Table 3, the main risk factors for CRI on the basis of published reports are summarized.

Empirical therapy for catheter-related bloodstream infection Febrile cancer patients with CVC need a prompt antibiotic treatment after obtaining blood cultures [1]. At the beginning, when catheter-related bloodstream infection (CRBSI) is suspected but not yet documented, the choice of antibiotic treatment is empirical, based on the principles of febrile neutropenia, i.e., the use of broad-spectrum antibiotic/s targeting Gram+ and Gram−, administered through the CVC [1, 2] (BII). The epidemiology of the center, together with the clinical condition of the patient and his/her

Table 2 Definition of catheter-related infection (CRI)

Exit site infections	Clinical signs of inflammation (redness, swelling, pain, bleeding) at the site of leakage of the CVC
Infections of the subcutaneous tunnel	Signs of inflammation along the subcutaneous way of CVC at a distance >2 cm from the point of discharge of the CVC
Infection of the port-à-cath pocket	Signs of inflammation in the pocket of the port +/− exudation or necrosis of the overlying skin
CVC-related bloodstream infections (CRSBI)	Bacteremia or fungemia in a patient with a CVC with clinical symptoms (fever, chills +/− hypotension) and detection of at least one positive blood culture from peripheral vein in the absence of other infectious sources The diagnosis is supported by: 1. Positive semiquantitative (> 15 cfu) or quantitative (>10^2 cfu) culture of the tip of the CVC removed and concomitant blood culture from peripheral vein (AII); 2. Concomitant positivity of quantitative blood cultures from CVC and peripheral vein with a 3:1 ratio (AII); 3. Different positivization time (dTTP) between blood cultures from CVC and peripheral vein (positivization of blood cultures from CVC at least 2 h before the peripheral one) [1–4, 8, 40] (AII) 4. Infection at the exit site or in the subcutaneous tunnel with positive blood cultures from CVC
Probable CVC-related infection	Resolution of the fever within 48 h after the CVC removal in a patient with positive blood cultures with fever resistant to therapy
Possible CVC-related infection	Bacteremia by pathogens typically causative of CVC infection (*S. epidermidis*, *S. aureus*, *Candida* spp.); bacteremia without detection of other infectious foci
Polymicrobial CRBSI	Sepsis with isolation of >1 pathogen from single blood culture or from two different blood cultures in 24 h [1] (AI)

Table 3 Main risk factors for catheter-related infection

Risk factor	Notes	Type of study	Recommendation
Immunosuppression and level of neutropenia [2]	The type and severity of immunosuppression are associated with increased incidence of CRI; the duration of neutropenia correlates with risk of septic complications and with mortality	Adult population Guidelines Onco-hematological population	BII
Underline disease [6, 39]	Hematological disease correlates with increased incidence of infectious complications due to more frequent catheter manipulation	Pediatric population Prospective, observational study Onco-hematological population	BII
The time of CVC in situ permanence [2, 41]	It is straight correlated to the risk of infectious complication	Adult population Guidelines, review	BII
Frequency of manipulation [2, 9]	The frequency of manipulation is straight correlated to the risk of infectious complication	Adult population Guidelines, review	BII
Children age [6, 39]	Children age <4–6 years correlates with risk of septic complications	Pediatric population Prospective study Onco-hematological population	BII
Number of CVC lumen [6]	Double lumen CVC is associated with increased incidence of CRI	Pediatric population Prospective study Onco-hematological population	BII
Use of the subclavian vein [42, 43]	The use of the subclavian vein correlates with a reduced risk of infectious complications	Adult population Review, observational study	BII
Microbial colonization of the skin [2, 41, 42, 44]	Microbial colonization of the skin at the point of connection or the junction of the CVC is associated with increased incidence of CRI	Adult/pediatric population Guidelines, review, observational study	BII
Parenteral nutrition (TPN) or blood components administration [2, 41, 42, 44]	Administration of TPN or blood components through the CVC is associated with increased risk of septic complication	Adult/pediatric population Guidelines, review, observational study	BII
Prolonged hospitalization [2, 41, 42, 44]	A prolonged hospitalization before the implantation of the CVC correlates with increased risk of infectious complication	Adult/pediatric population Guidelines, review, observational study	BII

recent medical history, is to take into account in the choice of the type of antibiotic and of its use alone or in combination with other antibiotics [1, 2] (BII). The empirical treatment generally involves the use of a third/fourth generation cephalosporin (ceftazidime, cefepime) or of a β-lactam β-lactamases antibiotic (piperacillin/tazobactam). The addition of an aminoglycoside (amikacin) is not mandatory but it is recommended in case of suspicion of infection by multidrug-resistant *Pseudomonas aeruginosa* or *Escherichia coli* [1–3, 14–18] (BII). The carbapenems (imipenem, meropenem) have a broad spectrum of action and are active against the extended-spectrum β-lactamases (ESBL) strain producers but their use is not indicated as first choice in all CRSBI for the risk to select carbapenemases producer strains. Glycopeptides (teicoplanin, vancomycin) are not indicated as first-line treatment, as its early use is not associated with improvement of survival [1, 2]. Its use as first-line treatment may be justified in case of high incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections or in patients with a medical history positive for MRSA or in case of clinical picture of severe sepsis or infection of the subcutaneous tunnel [1–3, 14, 15] (BII).

The antibiotic treatment has to be reassessed in case of persistence of fever after 72–96 h from the initiation of empiric therapy (BII) and modified according to the results of blood cultures and/or on the basis of clinical conditions of the patients. The duration of antimicrobial treatment in case of CRBSI is of 10–14 days [1–3, 19] (BII). In case of bacteremia complicated with thrombosis, endocarditis, and osteomyelitis, treatment should be continued for at least 6 weeks [1, 8].

The empirical use of an antifungal is not generally indicated as first choice in the treatment of CRBSI but may be suggested in cases of septic shock [1, 2, 20]. In patients with one or more risk factors for sepsis by *Candida*, such as total parenteral nutrition, previous prolonged broad-spectrum antibiotic treatment, previous or current colonization in multiple sites by *Candida*, or recent hematopoietic stem cells or solid organ transplant, it is recommended to associate to initial antibiotic treatment an antifungal treatment for *Candida* [1, 2] (BII). Echinocandins are the first choice antifungal treatment both in neutropenic and non-neutropenic patients for infections caused both by *Candida albicans* and *Candida* non-*albicans*. Second choice drugs are liposomal amphotericin B (LAMB) and voriconazole. Fluconazole is indicated for patients colonized by *Candida* fluconazole-sensitive strains or patients with a proven candidemia by *C. albicans* and no hemodynamic instability [1, 2] (BIII).

Exit site and tunnel infection An uncomplicated exit site infection in non-neutropenic patient, characterized by hyperemia and redness, is treated with topical antimicrobials chosen according to antibiotic susceptibility test [1] (BIII). In the neutropenic patient, or in presence of fever, purulent drainage or no response to topical treatment alone, an intravenous antimicrobial therapy together with topical treatment is indicated [1] (BII).

The infection of the tunnel of partially implantable CVC or of the Port-a-cath reservoir, requires the CVC removal and systemic antimicrobial treatment for at least 7–10 days [1] (BIII). Most of these infections are caused by Gram− (CONS). Therefore, it is indicated to include a glycopeptide in the initial antibiotic treatment until the causative germ is identified [1, 21] (BIII).

Targeted therapy Several studies, even in non-oncologic setting, showed the success of systemic treatment in the resolution of CRBSI without CVC removal [1, 4]. Several authors agree that in children with CRBSI it is generally worthy to start empirical treatment and to postpone the CVC removal, performing it only in case of non-response [1, 4] (BIII). Once the germ is identified, the empiric antibiotic therapy has to comply with the susceptibility test. The indications for targeted therapy are summarized in Table 4. Antibiotics are generally administered intravenously by slow infusion. An alternative method of administration is the intravenous continuous infusion. Antibiotics that exhibit a time-dependent bactericidal activity and 12–24 h stability in solution at room temperature, such as beta-lactams and glycopeptides, can be used by continuous 24-h administration. The rationale is to ensure high (>MIC) and continuous antibiotic concentrations systemically through the CVC, to avoid frequent CVC manipulation and rapid infusion that can generate septic emboli [22–25]. The use of β-lactams or glycopeptides in a 24-h continuous infusion through the CVC is indicated in case of bacteremia by Gram+ and Gram− drug-resistant or difficult to eradicate germs, where it is important to preserve the CVC. The ideal concentrations are <5 mg/ml vancomycin and <6 mg/ml ceftazidime [23–25] (BIII). This modality of infusion may also be used in case of CVC subcutaneous tunnel infections [21] (BIII).

Lock therapy Lock therapy is indicated in case of CVC colonization by CONS or other germs in patients with highly needed CVC. Lock therapy is indicated for 10–14 days in order to prevent the removal of CVC [26] (BII). In CRBSI, it can be used in association with systemic antibiotic for 7–14 days because it has been associated to a reduction of CRBSI recurrence. The renewal of the lock is suggested every 24–48 h, without serial monitoring with blood cultures [1, 5, 27–30] (BIII).

CVC removal The removal of CVC is indicated in cases of CRI by *S. aureus*, *P. aeruginosa*, mycobacteria or fungi, or in case of suppurative thrombophlebitis, endocarditis, and bacteremia unresponsive to 72–96 h of adequate targeted antibiotic therapy [1–3, 5, 9] (BII). In case of *Candida* infection, there are no studies that demonstrate the real benefit of early CVC removal, but all experts agree that success of therapy is associated with early CVC removal [31–33] (BIII). There are no studies to assess what is the optimal time for a CVC re-implantation. The CVC re-implantation requires at least two negative blood cultures, performed after appropriate antibiotic therapy (at least 5–7 days), to confirm the eradication of the pathogen [1, 3] (BIII).

In case of fungal infection, antifungal treatment should be continued for at least 14 days after the finding of the first negative blood culture, except for documented fungal localization in other sites [1].

Prophylaxis It is known that compliance with the rules of asepsis and proper handling of the CVC is the basis for the prevention of CVC-related infections [1, 2, 9, 19] (AI). No evidence is supported in the literature about the success of the systemic antibacterial prophylaxis in the reduction of the incidence of CRBSI in adult patients [9, 34]. In oncologic neutropenic adults and children, the administration of vancomycin/teicoplanin before the placement of a tunneled CVC does not significantly reduce the number of Gram + CRI [34]. The use of systemic antimicrobials is not routinely indicated before CVC insertion or during its use [34] (BI). In pediatric patients with severe neutropenia or fever, the use of short-term, prophylactic, systemic, preoperative therapy with intravenous glycopeptide may be considered (CIII). A wide variety of solutions containing antibiotics or antiseptics have been used for CVC irrigation (flushing) or

Table 4 Targeted therapy [1, 2, 8]

Pathogen	1st choice treatment	Alternative treatments	Notes
Gram + cocci			
Staphylococcus aureus			Recommended CVC removal and targeted antibiotic treatment for at least 4–6 weeks. Stop treatment at 14th day if infection is not complicated or in non-neutropenic patient, not transplanted and without comorbidities or risk factors such as diabetes and steroidal or immunosuppressive therapy. Lock therapy is indicated; CVC removal in case of no response or complicated infection
Methicillin-sensitive	Penicillin penicillinase-resistant	Cephalosporins, glycopeptide	
Methicillin-resistant	Vancomycin	Teicoplanin, daptomycin, linezolid, rifampicin + vancomycin, vancomycin + gentamicin	
Coagulase-negative staphylococci (CONS)			Generally benign clinical course, intravenous antibiotic treatment for 7–14 days and in persistently neutropenic patients for at least 7 days after the resolution of the fever
Methicillin-sensitive	Penicillin penicillinase-resistant	Cephalosporins, glycopeptide	
Methicillin-resistant	Vancomycin	Teicoplanin, daptomycin, linezolid	
Enterococci			Intravenous antibiotic treatment for 7–14 days, and in persistently neutropenic patients for at least 7 days after the resolution of the fever
Ampicillin-sensitive	Ampicillin sulbactam + aminoglycoside	Aminoglycoside + glycopeptide	
Ampicillin-resistant vancomycin-sensitive	Vancomycin or teicoplanin + aminoglycoside	Linezolid/daptomycin + aminoglycoside	
Ampicillin-resistant vancomycin-resistant	Linezolid/daptomycin		
Gram− bacilli			
Escherichia coli and *Klebsiella* spp.			In case of resistance to carbapenems, targeted treatment with colistin or aminoglycoside. In case of persistent fever and/or positive cultures, CVC removal indicated
ESBL neg	3rd-generation cephalosporins	Quinolone	
ESBL pos	Carbapenem	Quinolone	
Enterobacter species, *Serratia marcescens*	Carbapenem	Ciprofloxacin/cefepime	In case of persistent fever and/or positive cultures, CVC removal indicated
Acinetobacter species	Ampicillin sulbactam, carbapenem		In case of persistent fever and/or positive cultures, CVC removal indicated
Pseudomonas aeruginosa	3rd- to 4th-generation cephalosporins, carbapenem, piperacillin–tazobactam+/– aminoglycoside		In case of persistent fever and/or positive cultures, CVC removal indicated
Stenotrophomonas maltophilia	Cotrimoxazole	Colistin/tigecycline/ticarcillin–clavulanic acid	In case of persistent fever and/or positive cultures, CVC removal indicated
Candida spp.			
C. albicans	Fluconazole/echinocandins in neutropenic patients	Echinocandins/LAMB	CVC should be removed and antifungal therapy should be performed for 14 days from the first negative blood culture. The use of systemic antifungal therapy associated with lock therapy is still under investigation
C. non-*albicans*	Echinocandins	LAMB	

CONS coagulase-negative staphylococci

lock therapy for a period ranging from several hours to 24–48 h [34–38]. A recent meta-analysis on adult patients has showed that CVC flushing with vancomycin and low-dose heparin, compared to heparin alone, reduces the risk of CVC early infection (within 30 days) in high-risk patients [34]. In a randomized study, lock therapy with vancomycin has been shown to be more effective than flushing with normal saline or heparin [37] (AIII). As a low CRI rate has been documented in several pediatric studies using normal saline flushing, this expert panel recommends the use of vancomycin flushing solution for the centers that have a higher incidence of CRI by CONS or MRSA. [6, 17, 26, 39] (BII).

Conclusions

This is the first review about the management of CVC infectious complications that proposes specific indications about pediatric onco-hematological patients. Considering the small

number of well done study in pediatric age, many considerations in this paper are similar to the onco-hematological adult population. However, if compared to the adult age, in children a major indication is the importance of conservative, pharmacological therapy through CVC and the CVC remotion postponement due to the exiguity of venous access. The necessity to perform periferal vein cultures remains controversial in children, due to the younger age and the poor venous asset.

Other prospective randomized pediatric studies are necessary to obtain specific indications about the diagnosis of CVC infection and the time for remotion and replacement of the device.

Conflict of interest The authors declare that they have no conflict of interest.

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Risk of Seizures in Children Receiving Busulphan-Containing Regimens for Stem Cell Transplantation

Désirée Caselli [1], Anna Rosati [2], Maura Faraci [3], Marta Podda [4], Mimmo Ripaldi [5], Daniela Longoni [6], Simone Cesaro [7], Luca Lo Nigro [8], Olivia Paolicchi [1], Natasha Maximova [9], Maria Cristina Menconi [10], Ottavio Ziino [11], Maria Pia Cicalese [12], Stella Santarone [13], Francesca Nesi [14], Maurizio Aricò [1,15,*], Franco Locatelli [16], Arcangelo Prete [17] on behalf of the Bone Marrow Transplantation Working Group of the Associazione Italiana Ematologia Oncologia Pediatrica

[1] Pediatric Hematology Oncology and Domiciliary Care, Azienda Ospedaliero-Universitaria A. Meyer Children Hospital, Firenze, Italy
[2] Pediatric Hematology Oncology, Neurology, Azienda Ospedaliero-Universitaria A. Meyer Children Hospital, Firenze, Italy
[3] Pediatric Hematology Oncology, Hematopoietic Stem Cell Transplant Unit, IRCCS G. Gaslini, Genova, Italy
[4] Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy
[5] Pediatric Hematology-Oncology, A.O. Pausilipon, Napoli, Italy
[6] Pediatric Hematology-Oncology, University of Milano Bicocca, Ospedale San Gerardo, Monza, Italy
[7] Pediatric Hematology-Oncology, Policlinico di Verona, Italy
[8] Pediatric Hematology-Oncology, Policlinico di Catania, Italy
[9] Pediatric Hematology-Oncology, IRCCS Burlo Garofolo, Trieste, Italy
[10] Pediatric Hematology-Oncology, Azienda Ospedaliero-Universitaria Pisa, Italy
[11] Pediatric Hematology-Oncology, ARNAS Ospedale Civico, Palermo, Italy
[12] Pediatric Hematology, Paediatric Immunology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute and San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy
[13] Pediatric Hematology-Oncology, Pescara, Italy
[14] Pediatric Hematology-Oncology, Ospedale Sant'Anna, Turin, Italy
[15] Pediatric Oncology Regional Network, Istituto Toscano Tumori (I.T.T.), Firenze, Italy
[16] Pediatric Hematology-Oncology, IRCCS Bambino Gesù Children Hospital, Rome, Italy
[17] Pediatric Hematology-Oncology, University of Bologna, Bologna, Italy

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A B S T R A C T

Busulphan (BU) is associated with neurotoxicity and risk of seizures. Hence, seizure prophylaxis is routinely utilized during BU administration for stem cell transplantation (SCT). We collected data on the incidence of seizures among children undergoing SCT in Italy. Fourteen pediatric transplantation centers agreed to report unselected data on children receiving BU as part of the conditioning regimen for SCT between 2005 and 2012. Data on 954 pediatric transplantation procedures were collected; of them, 66% of the patients received BU orally, and the remaining 34%, i.v. All the patients received prophylaxis of seizures, according to local protocols, consisting of different schedules and drugs. A total of 13 patients (1.3%) developed seizures; of them, 3 had a history of epilepsy (or other seizure-related pre-existing condition); 3 had documented brain lesions potentially causing seizures per se; 1 had febrile seizures, 1 severe hypo-osmolality. In the remaining 5 patients, seizures were considered not explained and, thus, potentially related to BU administration. The incidence of seizures in children receiving BU-containing regimen was very low (1.3%); furthermore, most of them had at least 1—either pre-existing or concurrent—associated risk factor for seizures.

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INTRODUCTION

Busulphan (BU) is an alkylating agent, employed for over 30 years in a variety of conditioning regimens for stem cell transplantation (SCT) as an alternative to total body irradiation (TBI). Initially introduced as a palliative treatment for chronic myeloid leukemia in adults [1], it was then used as a myeloablative drug in association with cyclophosphamide [2]. BU was rapidly recognized as an effective conditioning regimen for a variety of malignant and nonmalignant hematologic diseases, providing a good alternative to TBI when it was administered at myeloablative dose (16 mg/kg) [3].

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* Correspondence and reprint requests: Maurizio Aricò, Coordinatore Rete Regionale Oncoematologia Pediatrica, Istituto Toscano Tumori, Viale Pieraccini 6, 50139 Firenze, Italy.
E-mail address: maurizio.arico@ittumori.it (M. Aricò).

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Recently, it has been used, in combination with melphalan and fludarabine, in the so-called reduced-intensity conditioning regimens in adults and children at a dosage of 3.2 or 6.4 mg/kg [4].

A limited degree of plasma protein binding allows BU, unlike other lipophilic alkylating agents such as melphalan, to easily cross the blood-brain barrier, thus achieving levels in cerebrospinal fluid that are similar to those in plasma [5,6]. Neurotoxicity was associated with BU in animals [7] and in humans, also favored by an altered blood-brain barrier [8]. The incidence of neurotoxicity after BU-based conditioning therapy is reported up to 10% in adults [9], and approximately 7% in children [10]. Vassal et al. reported that higher doses (600 mg/m^2 or 16 mg/kg) were associated with an increased probability of neurotoxic manifestations [5]. Generalized seizures are the main manifestation of BU neurotoxicity. They are more frequent in older patients and appear to be dose dependent, both in children and adults [6,10,11]. In adults, seizures typically occur in the third or fourth day of BU administration, probably as a result of drug accumulation [6,8,12]. Even without overt seizure activity, electroencephalogram abnormalities can occur in up to 60% of patients [11,13,14].

Various antiepileptic drugs (AEDs) have been used for seizure prophylaxis, including phenobarbital sodium, benzodiazepines (clonazepam, lorazepam), and phenytoin [15-19].

It is worth mentioning that between .5% and 1% of children in the general population experience a nonrecurrent, single, unprovoked convulsive episode [20]. Considering both the potential pharmacokinetic drug interactions between AEDs and BU [21] and the effective risk of BU-related seizures, we decided to re-evaluate the current standard practice of using of AEDs for seizure prophylaxis in patients receiving BU before hematopoietic SCT [22]. With the aim to evaluate the incidence of seizures in children treated with BU, we performed a retrospective analysis among the pediatric hematology-oncology centers of the Associazione Italiana Ematologia Oncologia Pediatrica.

MATERIALS AND METHODS

All of the Associazione Italiana Ematologia Oncologia Pediatrica (www.aieop.org) centers were invited to participate in a retrospective data collection on all children receiving BU as part of their conditioning regimen for SCT between 2005 and 2012.

Data on route of administration, drug monitoring, seizure-specific risks factors, neurological associated conditions, seizure prophylaxis, and occurrence of seizures were collected on a specific form and pooled.

Only seizures occurring during or soon after (up to 2 days) last BU administration were considered to be potentially related [15].

RESULTS

A total of 954 transplantations performed in pediatric patients were reported by the 14 participating centers. In 637 (66%) of them, BU was administered orally, whereas in the remaining cases BU was given i.v.. All patients received prophylaxis of seizures according to the local policies (Table 1).

Seizures were reported in a total of 13 patients (1.8%), 8 males and 5 females, with a median age of 9 years. The source for stem cells was autologous in 5 patients and allogeneic in 8. BU had been administered orally in 7 children and i.v. in 6.

Of the 13 reported episodes, only 5 occurred within the time interval during which BU is expected to be present in the peripheral blood, ie, on days -6 (n = 2), −5, −3, −1. The remaining episodes of seizures were observed between day +1 and day +86 from transplantation (Table 2).

Of the 13 patients who developed seizures, 3 had a history of epilepsy (or other seizure-related pre-existing condition); 1 had febrile seizures; 3 had a brain lesion documented by MRI, considered by the attending physicians as a possible cause for seizures; 1 developed seizures during documented severe plasma hypo-osmolality, in the presence of carbamazepine plasma levels exceeding the therapeutic range (22 mg/L; upper normal limit 10 mg/L). Thus, only in the remaining 5 patients the seizures were considered fully unexplained by any concurrent factor, and thus most likely related to BU administration (Table 2).

Table 1
List of Drugs Used, in Decreasing Order, for Prophylaxis of Seizures in Children Receiving Busulphan-containing Regimens for Stem Cell Transplantation

Drug (Route of Administration)	Schedule
Carbamazepine (orally)	10-15 mg/kg/d
Clonazepam (orally)	.1-.2/mg/kg/d
Valproate (orally)	10-20 mg/kg/d
Phenobarbital (orally)	3 mg/kg/d
Dintoine (orally)	5-10 mg/kg/d
Lorazepam (intravenously)	.03-.06 mg/kg/d
Lorazepam (orally)	.02-.05 (max. 2 mg) every 6 h, 30 min. before BU administration
Levetiracetam (orally)	20 mg/kg/d
Midazolam (intravenously)	.05 mg/kg/d

DISCUSSION

Acute symptomatic seizures are seizures occurring at the time of a systemic damage or in close temporal association with a documented brain damage [23]. The risk of experiencing an acute symptomatic seizure is 3.6% in an 80-year lifespan [24]. The frequency of BU-related seizures is reported in the literature in the range of 10% in the absence of specific prophylaxis [5,8,15]. This supported the development of anticonvulsant prophylaxis as a standard of care also in pediatrics [25]. Yet, this recommendation remains based on a few old studies, mainly retrospective, with very limited number of patients. Subsequent modifications in the transplantation regimens, supportive care, and monitoring strategies are, thus, not taken into account.

In the present study of a large pediatric cohort of 954 transplantations, in which all patients received a prophylaxis, (almost invariably orally, with carbamazepine accounting for over one third of the cases, and clonazepam, valproate, phenobarbital and dintoine in about 100 cases) the frequency of seizures was 1.3%. This finding of such a low incidence of seizures might be considered to lend some support to the continuous use of the prophylaxis. Yet, many concerns have been raised about the real utility of prophylaxis [26,27]. Some authors define this persistent recommendation as "an example of an outdated clinical practice that persist despite a paucity of good quality supporting medical evidence [22]." Furthermore, the use of different regimens of prophylaxis, with different drugs and different mechanisms of action [15] as observed also in our study, without any drug monitoring confirming the achievement of effective drug levels, or even by using drugs which are expected to become therapeutic in a longer time interval, might simply represent an additional variable in the peritransplant phase. Furthermore, in the absence of any prophylaxis, a comparably low incidence (1.8%) of seizures was observed in an old report of a small series of 57 children [10], whereas no seizures at all were observed in a large series of 344 adults, leading the authors to challenge the need for prophylaxis [22]. The current study of a large series of children shows a frequency of seizures comparable to that observed in untreated adults and children.

Table 2
Main Features of Thirteen Children Who Developed Seizures during SCT during or after a Busulphan-containing Preparative egimen

Case No.	Gender/ Age, yr	Stem Cells Source	BU Route	Day of Onset and Seizure Type	EEG	Prophylaxis and Notes
Likely Busulphan-Related						
1	F/1.3	UCB	Oral	−6; repeated episodes of ocular deviation	Irritative	Lorazepam i.v. since 24 h before BU; previous seizures; brain CT scan: left hypoplasia, scattered calcifications; possible ischemic origin.
2	M/13	ALLO	Oral	−6; generalized	Normal	Carbamazepine since 5 days before BU; hyponatremia (122 mEq/L); on therapy with carbamazepine, plasma levels exceeding therapeutic range (22 mg/L; range, 4 to 10).
3	M/16	Autologous	Oral	−5; generalized	Normal (on day -2)	Partial complex epilepsy, arachnoid cyst, on antiepileptic therapy
4	M/19	Autologous	Oral	−3; myoclonus	Bilateral spikes	Lorazepam i.v. since 24 h before BU
5	M/5.7	Autologous	Oral	−1; partial seizure evolving to generalized seizure	Irritative	Carbamazepine since 24 h before BU. Normal MRI
Case	Gender/ Age, yr	Stem Cells Source	BU Route	Seizure Type	EEG	Notes
Unlikely to be Busulphan-Related						
6	M/5	Autologous	i.v.	+1; generalized		Phenobarbital since 24 h before BU.
7	M/16	ALLO	i.v.	+1; generalized	Irritative	Epilepsy, on therapy with levetiracetam; clinical picture suggestive for PRES
8	F/13	MRD	i.v.	+1; generalized	NP	Dintoine since 24 h before BU. MRI: aspecific lesions
9	M/6	AUTO	Oral	+10; generalized		Dintoine since 24 h before BU.
10	F/9	MRD	i.v.	+13; generalized	Irritative	Dintoine since 24 h before BU. Previous encephalopathy; normal CT
11	F/6	PMRD	i.v.	+15; generalized	Irritative	Dintoine since 24 h before BU
12	F/9	MRD	i.v.	+2, +37; generalized	Irritative; frontal foci	Dintoine since 24 h before BU; MRI cortical-subcortical alterations, hyperintense on FLAIR
13	M/11	MRD	oral	+86; generalized		Dintoine since 24 h before BU. MRI: frontal nodal lesion (1 cm.), perilesional edema;

EEG, electroencephalogram; ALLO, allogeneic; UCB, umbilical cord blood; MRD; matched related donor; PMRD, partially matched related donor; CT, computed tomography; MRI, magnetic resonance imaging; PRES, Posterior Reversible Encephalopathy Syndrome; FLAIR, fluid attenuated inversion recovery.

Despite the prophylaxis, 13 patients in our study were reported to have developed seizures, which, at first evaluation, could have been considered as BU related. Yet, at a careful revision, the majority of them showed concurrent or associated factors, which could, per se, make those children at risk for seizures. In particular, 2 of them had a history of epilepsy and were on antiepileptic therapy (not prophylaxis), 1 had previous seizures with evident brain abnormalities at computed tomography scan, and another had febrile seizures; a fifth child developed seizures during inappropriate antidiuretic hormone secretion, documented by concurrent severe hyponatremia, associated with, or possibly related to, the concurrent high plasma levels of carbamazepine; these event may be considered to be potentially related [28]. Three additional patients had either a history of undefined encephalopathy or gross abnormalities at magnetic resonance imaging, which in 1 case had justified antiepileptic therapy with phenobarbital. Thus, of the total 13, only in 5 patients the seizures were finally considered as really unexpected. They are either males or females, ages between 5 and 19 years, undergoing autologous (n = 3) or allogeneic (n = 2) transplantations. Based on this finding, the proportion of patients who developed BU-related, otherwise unexplained seizures might be as low as .75%.

Another open issue, ie, potential interference with BU pharmacokinetic [21], could also be worth investigation, but this fell outside the scopes of this retrospective survey.

In conclusion, in the present large series of children, the proportion of children exposed to BU-containing regimen for SCT while receiving specific prophylaxis who develop BU-related seizures appears to be very low, in the range of less than 1%. Furthermore, anticonvulsants, as most drugs, may have side effects and can alter the pharmacokinetic of other drugs used during SCT [29,30]. Yet, whether children can be given BU without prophylaxis is a question, which cannot be answered with the present data. A prospective study on this issue appears warranted.

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THE JOURNAL OF PEDIATRICS • www.jpeds.com ORIGINAL ARTICLES

Single-Day Trimethoprim/Sulfamethoxazole Prophylaxis for *Pneumocystis* Pneumonia in Children with Cancer☆

Désirée Caselli, MD[1,*], Maria Grazia Petris, MD[2], Roberto Rondelli, MD[3], Francesca Carraro, MD[4], Antonella Colombini, MD[5], Paola Muggeo, MD[6], Ottavio Ziino, MD[7], Fraia Melchionda, MD[3], Giovanna Russo, MD[8], Paolo Pierani, MD[9], Elena Soncini, MD[10], Raffaella DeSantis, MD[11], Giulio Zanazzo, MD[12], Angelica Barone, MD[13], Simone Cesaro, MD[14], Monica Cellini, MD[15], Rossella Mura, MD[16], Giuseppe M. Milano, MD[17,**], Cristina Meazza, MD[18], Maria P. Cicalese, MD[19], Serena Tropia, MD[7], Salvatore De Masi, MD[20], Elio Castagnola, MD[21], and Maurizio Aricò, MD[1,***], on behalf of the Infectious Diseases Working Group of the Associazione Italiana Ematologia Oncologia Pediatrica****

Objective To determine whether a simplified, 1-day/week regimen of trimethoprim/sulfamethoxazole is sufficient to prevent *Pneumocystis (jirovecii [carinii])* pneumonia (PCP). Current recommended regimens for prophylaxis against PCP range from daily administration to 3 consecutive days per week dosing.

Study design A prospective survey of the regimens adopted for the PCP prophylaxis in all patients treated for childhood cancer at pediatric hematology-oncology centers of the Associazione Italiana Ematologia Oncologia Pediatrica.

Results The 20 centers participating in the study reported a total of 2466 patients, including 1093 with solid tumor and 1373 with leukemia/lymphoma (or primary immunodeficiency; n = 2). Of these patients, 1371 (55.6%) received the 3-day/week prophylaxis regimen, 406 (16.5%) received the 2-day/week regimen, and 689 (27.9%), including 439 with leukemia/lymphoma, received the 1-day/week regimen. Overall, only 2 cases of PCP (0.08%) were reported, both in the 2-day/week group. By intention to treat, the cumulative incidence of PCP at 3 years was 0.09% overall (95% CI, 0.00-0.40%) and 0.51% for the 2-day/week group (95% CI, 0.10%-2.00%). Remarkably, both patients who failed had withdrawn from prophylaxis.

Conclusion A single-day course of prophylaxis with trimethoprim/sulfamethoxazole may be sufficient to prevent PCP in children with cancer undergoing intensive chemotherapy regimens. This simplified strategy might have implications for the emerging need for PCP prophylaxis in other patients subjected to the increased use of biological and nonbiological agents that induce higher levels of immune suppression, such as those with rheumatic diseases. *(J Pediatr 2014;164:389-92)*.

Pneumocystis (jirovecii [carinii]) pneumonia (PCP) is an opportunistic infection first recognized by the middle of the 20th century. Its incidence has increased over the last several decades, owing to the wider use of immunosuppressive therapy in organ transplant recipients and in patients with cancer or congenital or acquired severe immune deficiencienciency.[1-3] Before the use of prophylaxis, up to 43% of children with cancer developed PCP.[4] It is known that asymptomatic or mild pulmonary infections, defined as colonization, are widely observed in the general adult population. Serologic studies have shown that primary *P jirovecii* infection (as defined by the development of antibody responses to antigens) is acquired in early childhood; 70%-90% of healthy children exhibit serum antibodies to the organism by age 2-3 years.[5]

Genetic and epidemiologic data on *P jirovecii* infections in Italy are scarce, limited to defined geographical regions and mainly regarding isolates from patients with HIV infection. Dimonte et al[6] investigated a cohort comprising 263 patients from

AIEOP	Associazione Italiana Ematologia Oncologia Pediatrica
PCP	*Pneumocystis (jirovecii [carinii])* pneumonia
SMX	Sulfamethoxazole
TMP	Trimethoprim

From the [1]Department of Pediatric Hematology-Oncology, Azienda Ospedaliero Universitaria Meyer Children Hospital, Florence, Italy; [2]Pediatric Hematology-Oncology, Padua, Italy; [3]Pediatric Oncology and Hematology, Lalla Seràgnoli Unit, University of Bologna, Bologna, Italy; [4]Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, Turin, Italy; [5]Pediatric Hematology-Oncology, Monza, Italy; [6]Pediatric Hematology-Oncology, Bari, Italy; [7]Pediatric Hematology-Oncology, ARNAS Civico, Palermo, Italy; [8]Pediatric Hematology-Oncology, Catania, Italy; [9]Division of Pediatric Hematology/Oncology, G Salesi Women's and Children's Hospital, Ancona, Italy; [10]Pediatric Hematology-Oncology, BMT Unit, Spedali Civili, Brescia, Italy; [11]Pediatric Hematology-Oncology, Ospedale Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; [12]Pediatric Hematology-Oncology, Institute for Maternal and Child Health - IRCCS Burlo Garofolo, Trieste, Italy; [13]Pediatric and Hematology-Oncology, University Hospital Azienda Ospedaliero Universitaria di Parma, Parma, Italy; [14]Pediatric Hematology-Oncology, Azienda Ospedaliera Universitaria Integrata, Verona, Italy; [15]Pediatric Hematology-Oncology, University Hospital, Azienda Ospedaliero Universitaria Policlinico, Modena, Italy; [16]Pediatric Hematology-Oncology, Cagliari, Italy; [17]Pediatric Oncology/Hematology, BMT Unit, Section of Hematology, Department of Internal and Experimental Medicine, University of Perugia, Perugia, Italy; [18]Pediatric Oncology Unit, Fondazione IRCCS Istituto Nazionaledei Tumori, Milan, Italy; [19]Pediatric Immunology, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy; [20]Epidemiology, Azienda Ospedaliero Universitaria Meyer Children Hospital, Florence, Italy; and [21]Infectious Disease Unit, Gaslini Institute, Istituto Giannina Gaslini, Genoa, Italy.

*Current affiliation: Dept. Medical Direction, Azienda Ospedaliero Universitaria Meyer Children Hospital, Florence, Italy.

**Department of Pediatric Hematology/Oncology, Ospedale Pediatrico Bambino Gesù, Rome, Italy.

***Pediatric Hematology Oncology Network, Istituto Toscano Tumori , Florence, Italy.

****A list of members of the Infectious Diseases Working Group of the Associazione Italiana Ematologia Oncologia Pediatrica is available at www.jpeds.com (Appendix).

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2 major hospitals; 38 immunocompromised patients, including 25 patients with HIV infection; and 225 immunocompetent patients by polymerase chain reaction amplification of the mtLSU-rRNA gene and found that 25.5% were positive.

More than 40 years ago, Hughes et al[4] documented successful prophylaxis with daily trimethoprim (TMP)/sulfamethoxazole (SMX) in pediatric oncology patients. At present, this procedure is considered the standard of care for children with an immune defect due to chemotherapy or prolonged corticosteroid therapy.[7-9] The finding of the efficacy of daily, but intermittent prophylactic dosing of TMP/SMX was recognized in numerous subsequent studies.[7-12]

Current recommendations for TMP/SMX dosing for PCP prophylaxis in immunocompromised patients are based on either daily or 3 consecutive days per week dosing.[4,12] A recent study reported intermittent dosing of TMP/SMX based on a regimen of 2 consecutive days per week, used routinely for PCP prophylaxis in pediatric patients with leukemia and lymphoma. This dosing regimen was derived from studies on bone marrow transplantation recipients.[10]

The aim of the present study was to assess whether a less-intensive, 1-day/week regimen is sufficient to prevent PCP. To explore this issue, we performed a prospective survey of the results of PCP prophylaxis in children treated at pediatric hematology-oncology centers of the Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP).

Methods

All of the AIEOP centers were invited to participate in this prospective survey of PCP prophylaxis. Only patients with newly diagnosed cancer in 2009-2011 requiring chemotherapy and for which PCP prophylaxis was considered indicated according to local policy were eligible for the study.

Data on the total number of patients treated in study years, the number of solid tumors vs leukemia/lymphoma, criteria for prescribing PCP prophylaxis, the schedule of prophylactic regimens, and the number of cases of PCP reported prospectively in this cohort were collected. Data were collected as part of the supportive therapy in the individual disease-related protocol, with the informed consent of the patients' legal guardians. The capture of all PCP cases was performed by each participating center by using individual patient records, local databases, and study-specific data collection forms.

The data were collected on a specific form and pooled. The cumulative incidence of PCP was calculated both overall and in the 3 subgroups defined by duration of PCP prophylaxis. The differences in the cumulative incidence among subgroups were calculated using the Gray test. The analysis was performed on an intention-to-treat basis. The main presenting features (sex and age), the type of cancer diagnosis (hematologic malignancy vs solid tumor) were compared in the different therapeutic groups.

Results

A total of 20 centers participated in the study by reporting data on all of their patients newly diagnosed and treated between 2009 and 2011. These centers reported 3 different dosing regimens for PCP prophylaxis. Eleven centers prescribed a 3-day/week prophylaxis regimen, with TMP 5 mg/kg/day divided into 2 doses (total dose, 15 mg/kg/week). Six centers used a 2-day/week regimen with TMP either 10 mg/kg/day divided into 2 doses in 2 centers (total dose, 20 mg/kg/week) or 5 mg/kg/day divided into 2 doses in 4 centers (total dose, 10 mg/kg/week). The remaining 3 centers used the 1-day/week regimen, including 1 center with TMP 10 mg/kg/day divided into 2 doses, 1 center with 5 mg/kg/day divided into 2 doses, and 1 center with a 2-day/week (5 mg/kg/day divided into 2 doses) regimen for solid tumors and a 1-day/week (10 mg/kg/day divided into 2 doses) regimen for leukemia/lymphoma (Table). PCP prophylaxis was prescribed during the entire chemotherapy program.

A total of 2466 patients were analyzed, including 1093 with a solid tumor, 1371 with leukemia/lymphoma, and 2 with primary immunodeficiency. Among these patients, 1371 (55.6%) received the 3-day/week prophylaxis, 406 (16.5%) received the 2-day/week regimen, and 689 (27.9%) received the 1-day/week regimen.

Overall, only 2 cases of PCP (0.08%) were reported from the participating centers (Table). Both were enrolled in the group of patients receiving 2-day/week prophylaxis. In both cases, the diagnosis of PCP was suspected based on the clinical and radiologic findings and supported by polymerase chain reaction of bronchial aspirate or nasopharyngeal swab. Both patients were hospitalized and treated with intravenous high-dose TMP/SMX, and subsequently recovered.

The cumulative incidence of PCP at 3 years was as follows: overall, 0.09% (95% CI, 0.00%-0.40%); 2 days/week prophylaxis, 0.51% (95% CI, 0.10%-2.00%). Comparisons of the cumulative incidence of PCP in the 3 groups were as follows: 1-day/week vs 2-day/week, $P = .074$ (Gray test); 2-day/week vs 3-day/week, $P = .012$; 1-day/week vs 3-day/week vs 2-day/week, $P = .002$.

Remarkably, both of the patients who failed prophylaxis (ie, developed PCP during the study period) were not receiving prophylaxis, 1 because of drug intolerance and the other because of nonadherence to PCP prophylaxis.

To exclude a favorable selection bias in the therapeutic groups, we compared the frequency of patients with leukemia/lymphoma, aged up to 14 years (ie, the characteristics of the 2 patients who failed) in the 1-day/week group and the 2-day/week and 3-days/week groups, and were found to be comparable for age and duration of follow-up (P not significant, t test). Furthermore, when patients with leukemia/lymphoma were subdivided by age subgroup and diagnosis, no difference in the incidence of PCP was observed (P not significant, Fisher exact test).

Discussion

Although PCP prophylaxis for pediatric hematology/oncology patients has become the standard of care, the optimal regimen and duration of therapy have not been defined. The cumulative incidence of PCP in the 20

Table. Distribution of 2466 patients with cancer or PID by schedule of PCP prophylaxis, and number of failures observed during the study period (2009-2011) in 20 pediatric hematology-oncology centers

Center and regimen applied	Study population, n: Solid tumors	Study population, n: Leukemia/lymphoma	Total number of patients (%); rate × 1000 (95% CI)	PCP cases, 2009-2011
3 days/wk; total TMP/SMX dose, 15 mg/kg/wk			1371 (55.6); 0.0 (0.0-2.7)	
0201	244	104		0
0601	15	35		0
0307	79	17		0
0801	8	41		0
0401	137	222		0
0402	30	42		0
0305	42	65		0
0603	43	57		0
1303	37	40		0
0318	0	2 (PID)		0
1502	46	65		
Total	681	690		0
2 days/wk; total TMP/SMX dose, 10-20 mg/kg/wk			406 (16.5); 4.9 (0.6-17.7)	
0501	2	44		2
1602	12	41		0
1501	37	71		0
0602	8	26		0
1308	31	0		0
0101	72	62		0
Total	162	244		2
1 day/wk; total TMP/SMX dose, 5-10 mg/kg/wk			689 (27.9); 0.0 (0.0-5.3)	
0302	0	211		0
1308	0	101		0
0901	31	58		0
0701	219	69		0
Total	250	439		0
Total	1093	1373	2466; 0.8 (0.1-2.9)	2

PID, primary immunodeficiency.

participating AIEOP centers during the 3 years of observation (2009-2011) was extremely low (0.09%). Because the empiric antibiotic therapy currently recommended by the AIEOP does not include TMP/SMX unless interstitial pneumonia is suspected and specific diagnostic tests are performed, we suppose that underestimation of PCP in this cohort, although possible, is unlikely. Our findings confirm that pediatric centers are highly committed to perform PCP prophylaxis, which results in effective protection for their patients.

Only 2 cases of failure of prophylaxis (ie, of PCP) were found in the study population. On individual reevaluation, both patients admitted that, despite the prescription based on intention to treat, they were not taking TMP/SMX. One patient was an adolescent who admitted to being nonadherent, and the other patient was considered "intolerant" to TMP/SMX, but was offered no alternative prophylactic regimen. Thus, although we have no data on the actual compliance with PCP prophylaxis, PCP was not detected in patients compliant with prescribed PCP prophylaxis on any dosing schedule.

During the 8 years preceding our study period (2000-2008), 6 cases of PCP had been recorded at the same participating centers (data not shown). The causes of these "failures" of prophylaxis were withdrawal by patient or parental decision (n = 3) and temporary hold of prophylaxis because of concurrent liver toxicity, treatment with another antibiotic, or graft-vs-host disease. Thus, nonadherence remains the sole reason for failure of PCP prophylaxis with TMP/SMX in children with cancer.[13] This concept should be kept in mind by the pediatric oncologists when considering differential diagnosis of interstitial pneumonia in their patients.

There are several patient-related factors not reported here that might have confounded our results, including, but not limited to, different levels of risk for PCP based on intensity of chemotherapy and severity of immunosuppression. Although the Gray test takes "competing risks" into consideration, these confounding factors that might affect the incidence of PCP should be evaluated or discussed. To address this issue, we have compared the main characteristics of the patients receiving single-day prophylaxis and those receiving more extended dosing schedules, and found no positive selection in terms of sex, age, diagnosis, or duration of follow-up. Furthermore, the lack of failure of PCP prophylaxis in all but 2 patients (nonadherence) makes these potentially confounding factors less relevant in this analysis.

Overall, our data confirm that PCP prophylaxis has been very successful in preventing PCP in a large cohort of children with cancer treated at the participating AIEOP centers. This result was achieved using TMP/SMX administration with different dosing schedules, ranging from 1 day to 3 days per week. Although the drug is administered orally and its cost is

very reasonable, this difference may be of interest. Because compliance remains the basis for the success of prophylaxis, and in turn lack of compliance is the main cause of failures, a simpler regimen with shorter duration (eg, with a single, specific day of the week, say on Sunday) may be expected to improve compliance. Available data from the literature suggest that based on the original report of continuous TMP/SMX administration,[4-7] attempts have been made to simplify the administration schedule. The first step was a regimen of 3 consecutive days per week, which was widely adopted for many years.[12] Recently, Agrawal et al[14] reported 87 patients treated twice daily for 2 consecutive days per week, with no proven case of PCP. That study was limited by relatively small patient numbers, however. Souza et al[15] reported a different prophylaxis based on twice-weekly TMP/SMX or weekly dapsone (a nonstandard option for PCP prophylaxis), and their findings with a 2-day/week regimen were comparable with earlier 3-day/week studies. Lindemulder and Albano[10] reported TMP/SMX (TMP 5 mg/kg/day) on 2 consecutive days, with no failures. Ohata et al[16] reported a higher dose (8 mg/kg/day) on 2 nonconsecutive days per week, with no failures. These data suggest that a twice-weekly regimen may be appropriate for common use; however, Agrawal et al[14] suggested that "once-weekly prophylaxis might be successful for some patients."

Because the use of TMP/SMX is associated with mild myelosuppression, which often affects delivery of chemotherapy, a simplified regimen with a lower cumulative dose also might have a reduced impact on chemotherapy, thus favoring more regular administration.[17]

Although the need for PCP prophylaxis in children with solid tumors may be considered less clear or even questioned by some authors, prophylaxis is considered a standard of care in children with hematologic malignancies. In the present study, 439 children with leukemia/lymphoma received a simplified regimen with once-daily TMP/SMX, and none developed PCP. Thus, this study provides suggestive evidence that a simplified, single-day prophylactic regimen with TMP/SMX may be sufficient to prevent PCP in children with either solid tumor or leukemia/lymphoma treated with a contemporary intensive chemotherapy regimen. Of note, the increased use of biological and nonbiological agents that induce higher levels of immune suppression can make this strategy of potential clinical interest for other patient populations as well, such as those with rheumatic diseases.[18,19] In addition, because of improved diagnosis and supportive care, an increasing number of children with congenital immune deficiency survive and thus remain at risk for PCP.[20] ■

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Reprint requests: Maurizio Aricò, MD, Istituto Toscano Tumori, Viale Pieraccini 6, I-50139 Firenze, Italy. E-mail: maurizio.arico@ittumori.it

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Appendix. Additional members of the Infectious Diseases Working Group of the AIEOP

Center	Members
Oncoematologia Pediatrica E Patologia Della Coagulazione Ospedale Regionale, Cagliari	Orofino Maria Graz, MD
Divisione Ematologia - Oncologia Pediatrica Clinica Pediatrica, Catania	La Spina Milena, MD
Dipartimento A.I. Oncoematologia SODC Tumori Pediatrici E Trapianto Di Cellule Staminali, Firenze	Tucci Fabio, MD
Servizio Di Oncologia Pediatrica Dipartimento Di Pediatria Il Ateneo Di Napoli, Napoli	Ripaldi Mimmo, MD D'amico Maria Rosaria, MD
Dipartimento Di Pediatria Universita' Di Padova Cattedra Di Oncoematologia Pediatrica, Padova	Calore Elisabetta, MD
Oncoematologia Pediatrica Fondazione Irccs, Policlinico San Matteo, Pavia	Baldanti Fausto, MD Decembrino Ninni, MD
Dipartimento Di Ematologia Clinica- Presidio Ospedale Civile Dello Spirito Santo, Pescara	Santarone Stella, MD
SC Di Oncoematologia Pediatrica Con Trapianto Di CSE, Ospedale SM Della Misericordia, AO Perugia, San Sisto-Perugia	Perruccio Katia, MD
S.C. Oncoematologia Pediatria E Centro Trapianti Ospedale Infantile Regina Margherita. P, Torino	Carraro Francesca, MD
U.O. Emato-Oncologia Pediatrica Universita' Degli Studi Di Trieste Osp.Le Infantile Burlo Garofolo, Trieste	Maximova Natalia, MD
Advisor. Oncoematologia Pediatrica Ospedale Bambino Gesu, Roma	Livadiotti Susanna, MD

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ORIGINAL ARTICLE

Central venous access devices in pediatric malignancies: a position paper of Italian Association of Pediatric Hematology and Oncology

Alessandro Crocoli[1], Assunta Tornesello[2], Mauro Pittiruti[3], Angelica Barone[4], Paola Muggeo[5], Alessandro Inserra[1], Angelo Claudio Molinari[6], Valeria Grillenzoni[7], Viviana Durante[8], Maria Pia Cicalese[9], Giulio Andrea Zanazzo[10], Simone Cesaro[7]

[1] General and Thoracic Surgery Unit, Department of Surgery, Bambino Gesù Children's Hospital, IRCCS, Rome - Italy
[2] Pediatric Oncology Unit, Vito Fazzi Hospital, Lecce - Italy
[3] Department of Surgery, "Agostino Gemelli" Catholic University, Rome - Italy
[4] Pediatric Hematology and Oncology Unit, Department of Pediatrics, Azienda Ospedaliero-Universitaria di Parma, Parma - Italy
[5] Pediatric Hematology and Oncology, Pediatric Clinic, Azienda Ospedaliero-Universitaria Policlinico, Bari - Italy
[6] Thrombosis and Hemostasis Unit, Giannina Gaslini's Children Hospital, Genova - Italy
[7] Pediatric Hematology Oncology, Azienda Ospedaliera Universitaria Integrata, Verona - Italy
[8] Pediatric Surgery Unit, University Policlinic Hospital, Modena - Italy
[9] Pediatric Immunology and Bone Marrow Transplantation Unit, San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), "San Raffaele" Scientific Institute, Milan - Italy
[10] Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", Trieste - Italy

ABSTRACT

Introduction: Treatment of pediatric malignancies is becoming progressively more complex, implying the adoption of multimodal therapies. A reliable, long-lasting venous access represents one of the critical requirements for the success of those treatments. Recent technical innovations—such as minimally invasive procedures for placement, new devices and novel materials—have rapidly spread for clinical use in adult patients, but are still not consistently used in the pediatric population.
Methods: The Supportive Therapy Working Group of Italian Association of Hematology and Oncology (AIEOP) reviewed medical literature focusing on new aspects of central venous access devices (VADs) in pediatric patients affected by oncohematological diseases.
Results: Appropriate recommendations for clinical use in these patients have been discussed and formulated.
Conclusions: The importance of the correct choice, management and use of VADs in pediatric oncohematological patients is a necessary prerequisite for an adequate standard of care, also considering the increased chances of cure and the longer life expectancy of those patients with modern therapies.

Keywords: Central venous catheter, Hematology, Oncology, Pediatric malignancies, PICC, Port

Introduction

In recent decades, the increasing use of venous access devices (VADs), either long term (LTVA) or medium term (MTVA), has provided a significant improvement in the treatment of children affected by oncohematologic diseases. According to the currently accepted terminology (1), LTVA is defined as a VAD with technical features which increase its stability in time, with an expected duration in the range of months and years: this category includes tunneled cuffed catheters and totally implantable venous ports. MTVA are VADs appropriate for a prolonged but not unlimited time (weeks or months) and for a discontinuous use (1): they include tunneled non-cuffed central catheters, peripherally inserted central catheters (PICCs) and midline catheters.

A reliable and long-lasting central VAD permits a safer and easier administration of chemotherapy, supportive drugs, hyperhydration and hypertonic solutions, such as total parenteral nutrition, than via the peripheral vein.

Placement of a central VAD can be performed by surgeons, anesthesiologists, intensivists, interventional radiologists, oncologists or even nurses, depending on the type of device and the choice of venous access. LTVA and MTVA can be placed with different techniques (venous cutdown, "blind" percutaneous venipuncture guided by anatomical

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Corresponding author:
Alessandro Crocoli, MD
General and Thoracic Surgery Unit
Department of Surgery and Centro Pediatrico
Bambino Gesù Basilicata
Bambino Gesù Children's Hospital
IRCCS, Rome, Italy
alessandro.crocoli@opbg.net

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landmarks, ultrasound-guided percutaneous puncture and cannulation of the vein). Placement of a central line always carries potential risks for the patient, with relevant differences in the type and severity of complication, depending on which vein, which technique and which device have been chosen.

The objective of this position paper, promoted by the Italian Association of Pediatric Oncology and Hematology (AIEOP) and developed by the Supportive Therapy Working Group (ST-WG) of AIEOP, is to provide practical recommendations for indication, choice, placement and removal of LTVA and MTVA in pediatric oncohematologic patients. The present document also includes recommendations on the prevention of early and late noninfective complications potentially related to VAD insertion.

Methods

The ST-WG developed this position paper in collaboration with the AIEOP working groups on Infection, Coagulation, Surgery and Nursing. A joint committee was established, including the ST-WG and one to two members from each of the other working groups. During a preliminary meeting, the topics and the literature search were defined. In the National Library of Medicine's MEDLINE database, we searched all clinical papers (clinical trials, retrospective studies, case reports, reviews and consensus papers) dealing with central venous access in pediatric cancer patients; only papers published between January 2001 and January 2014, in English language and available as full text, were considered. A first selection was performed by the ST-WG, excluding papers of poor relevance or not focused on MTVAs or LTVAs. All papers in full text were examined by each member of the joint committee. During a second meeting, the committee discussed the clinical evidence of the most relevant papers in each area (indication/choice of the device; choice of access/insertion technique; prevention of mechanical complications; removal of the device) and specific recommendations were suggested. The ST-WG prepared a final document which was approved by the joint committee in a third meeting.

The main recommendations are summarized in Table I and discussed below.

Results and Discussion

Indications for the Positioning of LTVA and MTVA; Selection of the Device

The choice of the VAD should be based on the patient's needs, taking into account duration and type of oncology/hematology treatment. There is general consensus that any chemotherapy treatment in pediatric patients should be delivered via an appropriate central venous access, as the risks associated with the infusion of vesicant or irritant drugs via a peripheral venous access are not acceptable. Many supportive therapies adopted in pediatric cancer patients, such as parenteral nutrition, also demand for a central line: most current guidelines state that peripheral lines (short and long i.v. cannulas; midline catheters) are inappropriate

TABLE I - Summary of AIEOP recommendations

Indications and selection criteria
For long-term treatments, ports should be preferred for intermittent use and cuffed tunneled catheters for frequent/continuous use.
For short- to medium-term treatments, PICCs are a valid option, but they should be inserted only when deep veins of the arm are of appropriate diameter.
There is no evident advantage of silicon vs. polyurethane.
Double-lumen VADs should be used only in selected cases.
Catheters inserted in the femoral vein for medium- to long-term treatments should be tunneled away from the groin.
Prevention of early complications
Ultrasound should be used for diagnosis of local pathologic conditions before the procedure and for diagnosis of procedure-related complications.
Ultrasound is necessary to assess vein patency before the procedure and to choose the vein most appropriate in terms of caliber, depth and position.
The internal diameter of the vein to be cannulated—as assessed by ultrasound—should be at least three times the external diameter of the catheter, so as to reduce the risk of venous thrombosis.
Ultrasound-guided venipuncture should be adopted, so as to reduce the risk of early and late complications.
Venous cannulation by surgical cutdown should be avoided.
"Blind" percutaneous puncture of central veins ("landmark" venipuncture) should be avoided.
In newborn and infants, internal jugular vein and brachio-cephalic vein are usually the first options for ultrasound-guided central venous access.
Soon after procedures potentially associated with pleural damage, pneumothorax should be excluded by ultrasound scan of the intercostal space.
A specifically and properly trained vascular team should be implemented, so to reduce both insertion-related and management-related complications.
Prevention of late mechanical complications
Noncuffed catheters should be permanently secured, preferably by a sutureless device.
Cuffed catheters should be secured for at least 2-4 weeks, preferably by a sutureless device.
Valved and nonvalved catheters have the same expected incidence of complications.
Removal
Local anesthesia/sedation is used to remove a cuffed tunneled device whereas general anesthesia is usually needed to remove a port.
After removing the device, skin should be closed with absorbable sutures and/or glue and an occlusive dressing should be applied for at least 48 hours.
Complete removal of the cuff is recommended.
Embolized fragments of the catheter should be removed by nonsurgical endovascular procedure.
In case of device removal because of infection, a new device should be inserted at different times and using a different venous approach.

AIEOP = Italian Association of Pediatric Oncology and Hematology; PICC = peripherally inserted central catheter; VAD = vascular access device.

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for delivering solutions with high osmolarity, or pH >9 or <5 (2). Short-term central venous access—such as nontunneled centrally inserted central venous catheters (CVCs)—are appropriate for intrahospital use and/or for a short period of time, but not as a long-standing venous access (1). Most children with malignancies require a reliable, stable venous access for discontinuous, extrahospital use: this is usually achieved by MTVA or LTVA, depending on the expected duration of need for the access.

Noncuffed PICCs are commonly considered as MTVA (1, 3); they may be a valid option for short- and medium-term durations of treatment, particularly when general anesthesia is contraindicated, since they can be inserted at bedside under local anesthesia and/or mild sedation (4-7). Though currently considered appropriate particularly for medium-term venous access (3), there is a growing evidence—particularly in adult cancer patients—that they might be used for a long period of time (8, 9).

Considering LTVA, there is no striking difference in terms of general clinical performance between tunneled cuffed catheters (such as Broviac, Hickman, Leonard, cuffed Groshong, ProLine) and totally implanted venous ports, although in children who require frequent venous access for blood sampling, parenteral nutrition and complex intravenous therapies, such as those eligible for high-dose chemotherapy and hematopoietic stem cell transplant, tunneled cuffed catheters are usually preferred. On the other hand, children requiring less intensive support and undergoing chemotherapy schedules with a 3-6 week interval, or adolescents, who may not accept an external device, are eligible for a port. In other words, venous ports are recommended for prolonged but intermittent vascular access while tunneled cuffed catheters are recommended for prolonged but frequent/continuous vascular access (10-12). Cuffed PICCs are now available and may have a future role as LTVAs, as an alternative option to centrally inserted tunneled cuffed catheters.

Choice of material

MTVAs and LTVAs are made of catheters of different materials, either silicon or polyurethane. There is no evidence of any difference between silicon and polyurethane in terms of risk of infective and thrombotic complications in the adult or in the pediatric population (13, 14). Silicon catheters have traditionally been the first choice for LTVA in pediatric patients (11, 15-18), although polyurethane catheters—and specially power injectable polyurethane catheters, which are made of third-generation polyurethanes—are as biocompatible as silicone catheters but less fragile; also, they are compatible with higher flow rates and are ideal for injection of contrast medium (19).

Table II summarizes the main types of external long-term and medium-term CVCs.

Caliber of catheter

The outer caliber of the catheter should be decided on the basis of the inner diameter of the vein, so as to prevent venous thrombosis (20). The caliber of vein should be measured by ultrasound scan (21). Ideally, the outer diameter of the catheter should be equal or smaller than one third of the internal diameter of the vein: for example, a 3 Fr (=1 mm) catheter is appropriate for a vein whose diameter is 9 Fr (3 mm) or larger (13, 16, 22, 23).

TABLE II - Central venous catheters commonly used in the pediatric population

	Material	Tip	Lumen	French
Long term (tunneled, cuffed external VADs)				
Broviac	Silicon	No valve	1	2.7, 4.2, 6.0, 7, 9.6
Hickman	Silicon	No valve	2	7, 12
Leonard	Silicon	No valve	2	10
Groshong	Silicon	Distal valve	1 or 2	5, 5, 7 8, 9
ProLine	P.I. PUR	No valve	1 or 2	5, 6
Medium term (<100 d)				
Silicon PICC	Silicon	No valve	1 or 2	3, 4, 5
Groshong PICC	Silicon	Distal valve	1 or 2	4, 5
Solo, PASV PICC	P.I. PUR	Proximal valve	1 or 2 or 3	3, 4, 5, 6
PASV PICC	PUR	Proximal valve	1 or 2	4, 5
PUR PICC	PUR	No valve	1 or 2	4, 5
P.I. PUR PICC	P.I. PUR	No valve	1 or 2 or 3	3, 4, 5, 6

PASV = pressure-activated safety valve; P.I. = power injectable; PICC = peripherally inserted central catheter; PUR = polyurethane; VAD = vascular access device.

Double-lumen catheters, either LTVA or MTVA, are indicated in patients undergoing hematopoietic stem cell transplantation, in critically ill patients and in any patient needing chronic infusion of noncompatible solutions, such as the case of pediatric patients candidate to both parenteral nutrition and chemotherapy (12, 24, 25), although some data suggest that the use of double-lumen devices might be associated with a higher risk of infection compared to single-lumen devices (18).

Choice of the vein

Central lines are characterized by the location of the tip of the catheter close to the cavo-atrial junction, either in the lower third of the superior vena cava or in the upper part of the atrium: this can be achieved by cannulation of either central or peripheral veins. For centrally inserted VADs, veins on the right side are preferred, so that the catheter may follow a direct path toward the atrio-caval junction. Data in adult patients suggest that the insertion on the right side may be associated with a lower incidence of thrombosis (26), although pediatric data in this regard are not available. In the preultrasound era, the internal jugular vein was considered the first choice, in terms of anatomical location and accessibility, while the "blind" puncture and cannulation of the subclavian vein was considered to be

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more dangerous, especially if the patient was a newborn or infant. In the ultrasound era, many different options for central venous cannulation are now available (internal jugular, external jugular, brachio-cephalic, subclavian and axillary veins) (21). The most appropriate vein should be chosen after proper ultrasound examination, considering the caliber of the vein and the potential risk associated with its cannulation (13, 21).

PICCs can be placed in any deep vein of the arm between the elbow and axilla (brachial vein, basilic vein, axillary vein and even cephalic vein in selected cases), but since the minimum size of the PICC is 3 Fr there is a limited indication in newborns and infants due to the smaller venous caliber (13, 19). There is no clear recommendation as regards choosing left vs. right side, while the deep veins of the upper arm (brachial and basilic veins, which can be cannulated by ultrasound guidance) should be preferred over the superficial veins of the antecubital fossa (2). Side, vein and puncture site should be decided after proper ultrasound scan of the vasculature of the arm (21). The presence of veins of insufficient diameter (such as <3 mm or <4 mm when, respectively, a 3 Fr or 4 Fr catheter is required) is a contraindication to PICC insertion.

LTVA (tunneled cuffed catheters and ports) as well as MTVA (tunneled noncuffed long catheters) are sometimes also inserted by the femoral access, with the tip of the catheter in the upper portion of the inferior vena cava: this can be a suitable option in patients with superior vena cava obstruction syndrome. The most relevant complication of femoral access is infection: should this route be chosen for a LTVA or MTVA, tunneling has to be performed away from the groin along the thigh with exit site on the side of the knee or upward to the periumbilical region, so as to reduce the risk of infection by germs originating from the groin (17, 19, 23).

Placement of LTVA and MTVA in pediatric patients

Before the introduction of ultrasound guidance in clinical practice, placement of centrally inserted catheters by "blind" venipuncture or venous cutdown had been widely used in pediatric patients.

"Blind" puncture of central veins is associated with the risk of failure, repeated punctures, accidental arterial injury and pneumothorax due to accidental pleural damage. Accidental damage to the pleura is significantly more frequent after subclavian than after jugular venipuncture (27). A peculiar complication is the so-called pinch-off syndrome, a compression of the catheter between the first rib and the clavicle resulting in malfunction, obstruction, fissure and/or fracture and embolization of a catheter fragment: this syndrome is exclusively associated with "blind" infraclavicular cannulation of the subclavian vein (12, 22, 23).

The use of the surgical technique for the isolation and cannulation of the vein (venous cutdown) is associated with a relevant incidence of tissue trauma, failure and local complications even in experienced hands. Vessel thrombosis occurs more frequently, because of the dissection of tissues and the direct section and ligation of the vessel. Also, the surgical isolation of the vein entails a higher risk of infection at the site of entry of the catheter (2, 28, 29).

In adult patients, the use of ultrasound guidance to select and puncture the vein is clearly associated with a significantly higher rate of success and a significantly lower incidence of mechanical, infective and thrombotic complications if compared to surgical "cutdown" or "blind percutaneous techniques" (21). Ultrasound permits visibility of the progression of the needle in the target vessel, reducing the likelihood of accidental arterial puncture or accidental damage to nerves and other surrounding structures (21).

Though the evidence in pediatric patients is still limited, most studies suggest that ultrasound guidance should become the state of the art of venipuncture in children and neonates, as it is already in adults (13, 30-32). The most important advantage of ultrasound is to allow the choice of the most appropriate vein after a scan of all possible options (21). By ultrasound guidance, puncture and cannulation of the vein are quicker and easier as well as associated with fewer complications; moreover, the reduced invasiveness of the procedure consistently reduces the risk of infection at the insertion site and the risk of catheter-related thrombosis (13, 30, 33-36).

Also, ultrasound permits real-time diagnosis of preexisting vascular anomalies (malformations, anatomic variants or thrombosis) and of early complications (hematoma postinjection, extravasation, dissection of the vessel or stenosis, pneumothorax, etc.) (12, 37).

In patients with a previous placement of a venous access or other risk factors for venous thrombosis (surgery of the neck or superior vena cava syndrome), ultrasound allows the assessment of the patency of the vessel to be cannulated (12, 21).

The central veins in adults and children that can be cannulated under ultrasound guidance are the internal jugular vein, the external jugular vein (in its deeper path), the brachial-cephalic, subclavian (usually by a supraclavicular approach), the axillary vein (by infraclavicular approach) and the cephalic vein (in the infraclavicular region). In newborns and infants, the only veins of significant size apt to be cannulated are usually the internal jugular and brachio-cephalic (13, 31).

After the ultrasound-guided puncture of the vessel, a guidewire is inserted and directed to the cavo-atrial junction. In neonates and infants, ultrasound is also useful to guide the wire into the superior vena cava toward the right atrium; the final position of the tip can be verified by echocardiography. The guidewire should never be inserted to a length superior than the distance between puncture site and right atrium, since even the softest guidewire may damage the heart wall or provoke dangerous arrhythmias. A guidewire can be advanced to the inferior vena cava only if the maneuver is performed under fluoroscopic guidance, but this is not routinely recommended and it might be risky, especially in neonates and infants. After the insertion of the guidewire—as all MTVAs and LTVAs are currently inserted by the modified Seldinger technique—a peel-away introducer is advanced over the guidewire until the lower portion of the superior vena cava. The guidewire is removed and the catheter is threaded through the introducer.

The optimal position of the catheter tip is at the junction between the right atrium and the superior vena cava (1, 2). The tip location should be ideally verified in real time during the procedure (by fluoroscopy, by intracavitary electro-

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cardiogram (ECG) or by echocardiography) or—as a second option—soon after the procedure (by chest x-ray or by echocardiography). In the case of femoral access, the recommended level of the catheter tip is in the inferior vena cava below the renal veins. Before starting to use an LTVA or MTVA, the correct position of the tip must be verified and documented in the medical record (2, 21, 33, 34). Intracavitary ECG and/or echocardiography is safer and more accurate than fluoroscopy for verifying tip location and is becoming increasingly used in clinical practice, although the experience in pediatric patients is still limited (38, 39).

In the case of subclavian vein access, ultrasound examination of the intercostal spaces is a suitable examination for the early diagnosis of a pneumothorax secondary to the procedure. Immediate ultrasound scan of the pleural space for detecting the "sliding sign" is needed in any difficult puncture of the subclavian or axillary vein, and particularly if the patient complains of shortness of breath, discomfort or pain worsening after catheter placement; chest x-ray is less accurate and less immediately available in this regard (21, 40).

Proper training of the vascular team (41) and proper choice of methods and materials (13) are the key factors in reducing operative time and risk of intra- and postprocedural complications. When the same team is also responsible for the management of the venous access, late complications are also reduced (37).

Prevention of late mechanical complications of CVC

The more frequent late mechanical complications are dislocation of the catheter, migration of the tip, lumen occlusion and catheter rupture. All these complications are clinically associated with a malfunction of the device, often as a failure to draw from and/or to infuse through the device. Dislocation is the leading cause of premature loss of external venous access, especially in younger children (11). Dislocation can be minimal (less than 1-2 cm), partial (more than 2 cm) or complete (complete removal of the device). All noncuffed central catheters—both tunneled and nontunneled—should be permanently secured to the skin by an adequate sutureless device (29), although securement by a sutureless device is also needed for cuffed tunneled catheters, at least in the first 3-4 weeks after placement. Management of the exit site should be performed by trained personnel and in accordance with the characteristics of the VAD and the type of dressing used. Sutures should be used only in very selected cases, that is, when attachment of the sutureless device to the skin does not appear to be reliable. A new securement device which is apparently more effective than sutures in preventing dislocation consists in a device which anchors the catheter directly to the subcutaneous tissue (42, 43), although clinical studies with this device in pediatric patients are not yet available.

The tunneled cuffed catheters have a Dacron cuff, which requires 2 to 4 weeks to ensure a proper securement to the subcutaneous tissue of the patient (12, 13, 44). The cuff must be placed inside the tunnel at least 2 cm far from the exit site, as recommended by the manufacturer. A cuff inappropriately placed too close to the exit site is a well-recognized cause of dislocation of tunneled cuffed catheters (8).

Catheter fixation to the muscular fascia with nonabsorbable braided sutures has also been used for reducing the risk of dislocation in cases where a delay of the healing process and cuff anchoring is expected (prolonged use of corticosteroids, Omenn syndrome, epidermolysis bullosa) (13). The new sutureless device anchoring the catheter to the subcutaneous tissue (42, 43) will most likely make these surgical securement obsolete.

The reservoir of venous ports is sometimes secured to the underlying muscle fascia (10), although this is not considered to be mandatory in all cases (26).

The migration of the tip is another common mechanical complication that can occur even without external dislocation of the catheter and is more frequent with silicon catheters, due to the softness of the material. It is often associated with inappropriately "high" position of the tip (i.e., when the tip does not enter the lower third of the superior vena cava).

The occlusion of the lumen is more frequent with smaller caliber catheters and/or when proper protocols of flushing and locking are not adopted. Valved catheters are available for both MTVA and LTVA, although there is no evidence that valved catheters (either with distal or with proximal valves) may be associated with a lower risk of occlusion (or of any other complication) (45-48). Lumen occlusion is basically related to the local policies of flushing and locking the VAD, rather than to the technique of insertion (2, 15).

Mechanical lesions, breakage and fracture of the device—particularly of PICCs—are significantly more frequent with silicon than polyurethane catheters. The "pinch-off" syndrome has been a frequent cause of catheter malfunction and fracture in the past, although it is bound to disappear in the ultrasound era, as it was invariably associated with the "blind" puncture of the subclavian vein by infraclavicular approach.

Removal of the CVC

Removal of MTVA or LTVA must be performed by experienced personnel, in an appropriate setting. Noncuffed catheters such as PICCs may be easily removed at the patient's bedside, whereas the removal of an LTVA requires proper aseptic conditions (operating or procedure room). Before removing a tunneled cuffed catheter, the cuff should be located by palpation or by ultrasound. If the cuff is placed quite distant from the exit site, removal may require a new incision of the overlying skin (49, 50).

In pediatric patients, local anesthesia/sedation is used to remove a cuffed tunneled device whereas general anesthesia is usually needed to remove a port (49-51).

After removing the device, the exit site must be compressed for at least 5 minutes to reduce local bleeding. The exit point or points must be closed with absorbable sutures and/or glue; an occlusive dressing should be applied for at least 48 hours to avoid the risk of air embolism (49, 50).

The complete removal of the cuff is necessary, since retained cuffs or cuff fragments may become a source of infection, create false radiological images or cause unsatisfactory cosmetic results (49-51).

In the case of catheter embolization, the removal of the fragment usually requires a nonsurgical endovascular procedure by an interventional radiologist (22, 52).

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In case of loss of the catheter due to complete dislocation, the VAD can be repositioned in the same vein. In case of infection, the procedures of VAD removal and repositioning are carried out at different times to avoid the risk of infecting the new device, and preferentially using a different venous access (53).

Conclusions

As far as we know, this is the first position paper reviewing and discussing the issues of choice, placement and removal of LTVAs from the perspective of oncologic pediatric patients. Despite the referenced literature, there is far less evidence for children than for adults. The use of ultrasound guidance is an important innovation to reduce invasiveness and lower the incidence of both early and late complications. The investment needed for adequate staff training, and the acquisition of appropriate materials and appropriate methodologies is counterbalanced by an improvement in patient safety and a lower morbidity and mortality. In pediatric oncohematology patients these results are even more important in the light of an increased chance of cure and a longer life expectancy.

Disclosures

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Chapter 5

“Clinical and biological characterization of patients with immunodeficiencies”

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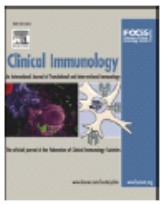

Human *IL2RA* null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity

Kevin Goudy [a, 1], Didem Aydin [a, 1], Federica Barzaghi [a, b], Eleonora Gambineri [c], Marina Vignoli [c], Sara Ciullini Mannurita [c], Claudio Doglioni [b, d], Maurilio Ponzoni [d], Maria Pia Cicalese [e], Andrea Assanelli [e], Alberto Tommasini [f], Immacolata Brigida [a], Rosa Maria Dellepiane [g], Silvana Martino [h], Sven Olek [i], Alessandro Aiuti [a, e], Fabio Ciceri [b, e], Maria Grazia Roncarolo [a, b, e], Rosa Bacchetta [a, e,*]

[a] *San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy*
[b] *Vita-Salute San Raffaele University, Milan, Italy*
[c] *Department of Science for Woman's Health, Anna Meyer Children's Hospital, Florence University of Florence, Italy*
[d] *Anatomo-Pathology Unit Ospedale San Raffaele IRCCS, Milan, Italy*
[e] *Pediatric Immuno-Hematology Unit and BMT Unit, Ospedale San Raffaele IRCCS, Milan, Italy*
[f] *Burlo-Garofalo IRCCS, Trieste, Italy*
[g] *Dipartimento di Pediatria, IRCCS Fondazione Policlinico, Università di Milano, Milan, Italy*
[h] *Department of Pediatrics, University of Turin, 10126 Turin, Italy*
[i] *Ivana Türbachová Laboratory of Epigenetics, Epiontis GmbH, Berlin, Germany*

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Abstract Cell-surface CD25 expression is critical for maintaining immune function and homeostasis. As in few reported cases, CD25 deficiency manifests with severe autoimmune enteritis and viral infections. To dissect the underlying immunological mechanisms driving these symptoms, we analyzed the regulatory and effector T cell functions in a CD25 deficient patient harboring a novel *IL2RA* mutation. Pronounced lymphoproliferation, mainly of the $CD8^+$ T cells, was detected together with an increase in T cell activation markers and elevated serum cytokines. However, Ag-specific responses were impaired *in vivo* and *in vitro*. Activated $CD8^+STAT5^+$ T cells with lytic potential infiltrated the skin, even though $FOXP3^+$ Tregs were present and maintained a higher capacity to respond to IL-2 compared to other T-cell subsets.

* Corresponding author at: San Raffaele Telethon Institute for Gene Therapy, Via Olgettina, 58, Milan 20132, Italy. Fax: +39 02 26434668.
E-mail addresses: bacchetta.rosa@hsr.it, rosa.bacchetta@hsr.it (R. Bacchetta).
[1] Authors contributed equally to the work.

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Thus, the complex pathogenesis of CD25 deficiency provides invaluable insight into the role of IL2/IL-2RA-dependent regulation in autoimmunity and inflammatory diseases.
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1. Introduction

The interleukin-2 (IL-2) receptor is formed by the α (IL-2RA, CD25) [1], β (IL-2RB, CD122) [2] and γ_{common} (IL-2RG, CD132) [3] subunits, and plays a vital role in maintaining the immune system. Among the IL-2 receptors, CD25 is a unique subunit that exclusively binds IL-2, while CD132 binds the common γ_c family cytokines (IL-4, IL-7, IL-9, IL-15 and IL-21), and the CD122 subunit binds IL-15. CD25 is constitutively expressed at high levels by $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs), and enables them to be the first responders to IL-2 during an immune response [4] and promotes the transcription of FOXP3 by amplifying IL-2 signaling in a STAT5-dependent fashion [5]. Interestingly, single nucleotide polymorphism (SNP) studies of the *IL2RA* gene have been associated with several forms of autoimmunity [6–10] demonstrating that IL-2 signaling via CD25 is an important axis in regulating tolerance. CD25 is also critical for effector T cell expansion in response to IL-2 immediately after antigenic stimulation. Although both $CD4^+$ and $CD8^+$ T cells up regulate CD25 and IL-2RB upon activation, $CD8^+$ T cells are more susceptible to IL-2 stimulation, probably due to their higher level of IL-2RB expression both in mice [11] and humans [12,13].

The immunological consequence resulting from the loss of CD25 has been ill-defined in man. Roifman's group was the first to describe a CD25 deficient patient who suffered from chronic infections and severe autoimmunity [14] resembling Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, caused by mutations in *FOXP3* gene [15]. This IPEX-like patient possessed a translation frameshift mutation in the *IL2RA* gene ablating its expression. Similarly, a second report described a patient with a different frameshift mutation in the *IL2RA* gene leading to a CD25 null phenotype with comparable clinical manifestations [16].

Here we describe the immunological findings of a patient carrying an *IL2RA* mutation not previously reported, selectively abrogating CD25 cell surface expression. Our results show, for the first time in human, the complex immunopathology associated with CD25 deficiency, and reveal a distinct pathogenetic mechanism of immune dysregulation.

2. Material and methods

2.1. *IL2RA* molecular analysis

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. PCR for each of the 8 exons of the human *IL2RA* gene (including exon/intron boundaries) was performed using PCR techniques as previously reported [17] and sequence conservation analysis of mutations was performed using PolyPhen, SIFT and SNPs3D tools.

2.2. Flow cytometry

PBMCs were isolated using Lymphoprep (Axis-shield) density gradient centrifugation. Surface Ab staining was performed for 30 min on ice in the absence of light using a 2% bovine serum albumin PBS mixture. Cells were washed and fixed with either 2% paraformaldehyde (Pierce) for later acquisition or with FOXP3 perm/fix buffer (eBioscience) to be further stained for FOXP3 or Ki67 The following Abs (all antibodies purchased from BD Biosciences unless otherwise noted): CD4 (SK3), CD8 (SK1), CD25 (2A3; M-A251), CD45RA (HI100), CD49d (L25), CD62L (SK11), CD69 (FN50), CD122 (MIKB2), CD132 (TUGh4), Ki67 (B56), FOXP3 (eBioscience PCH101), HLA-DR (L243), FASL (NOK-1), and HELIOS (22F6) (Biolegend).

2.3. T cell line generation and stimulation

Healthy donor cell lines were generated by stimulating 1×10^6 PBMCs with PHA 1 μg/ml (Sigma) in X-Vivo media (Biowhitaker) containing 5% human serum (Biowhitaker), 1% penicillin and streptomycin (Lonza), IL-2 (40 U/ml, Proleukin (Novartis)). On days 9, 14 and 20 the cells were washed and plated in the presence of IL-2 (100 U/ml), IL-7 (10 ng/ml), and IL-15 (10 ng/ml). For the CD25 deficient patient, $CD4^+$ T cells were enriched using $CD4^+$ T cell negative selection beads (Miltenyi) and cultured with IL-2 (100 U/ml), IL-15 (10 ng/ml), IL-7 (10 ng/ml). Cells were washed and restimulated with the same conditions on days 7, 11, and 20. On day 24, cells were washed and stimulated in 24 well plates (Corning) containing plate bound anti-CD3 (10 μg/ml) (BD Pharmingen) and anti-CD28 (1 μg/ml) (BD Pharmingen) in the presence or absence of IL-2 (100 U/ml) and IL-15 (10 ng/ml) for 6 h.

2.4. Measurement of sCD25

Levels of sCD25 were evaluated using a commercially available ELISA kit (BD Pharmingen). To measure sCD25 from activated cells, PBMCs (1×10^5) were stimulated for 72 h in complete RPMI (Biowhitaker) with plate-bound anti-CD3 (OKT3) (10 μg/ml) and soluble anti-CD28 (2 μg/ml) in the presence or absence of IL-2 (1000U/ml), TPA (Sigma)/Ionomycin (Sigma), or left unstimulated.

2.5. Phospho flow cytometry

To determine the phosphorylation (p) status of STAT3 and STAT5 after cytokine stimulation, a barcode technique was

employed as previously described [18]. Briefly, fresh PBMCs were rested overnight before stimulation with IL-2 (Low 10 U/ml, Med 100 U/ml, Hi 1000 U/ml), IL-15 (10 ng/ml), or IL-10 (10 ng/ml) for 0, 10, or 30 min. At the appropriate time point, the cells were fixed with 1.6% electron microscope grade paraformaldehyde (Pierce) for 10 min at 37° and then washed and permeabilized with 100% methanol (Sigma) for 10 min on ice. After washing, barcoding of the cells was performed using pacific blue succinimidyl ester (Invitrogen) suspended in PBS for 30 min. After washing, the individual wells were pulled into one tube and stained simultaneously with surface and intracellular-directed antibodies (CD4, CD8, pSTAT3 (pY705, 4/P-STAT3), pSTAT5 (Y694, clone 47) (BD Pharmingen)) for 30 min at room temperature. All samples were acquired on a FACScanto flow cytometer (BD Pharmingen).

2.6. Cytokine detection

To quantify cytokine levels in sera and plasma, the Bio-Plex protein array system was used according to the manufacturer's protocol. Levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), IL-17, TNF-α, and IFN-γ cytokines (BioRad) were analyzed using the Luminex system (Luminex) and concentrations of each cytokine were obtained using Bio-Plex Manager version 3.0 software (Luminex).

2.7. Microscopy

Following informed consent, skin biopsies were taken under local anesthetic and divided for microscopic analysis. For light microscopy, samples were formalin-fixed, paraffin embedded and sectioned at 4 μM for staining. Skin biopsies for immunofluorescence were mounted in OCT compound (Tissue Tek) and immediately snap-frozen in liquid nitrogen. Cryostat sections (5 μm) were mounted on to microscopy slides (Fischer) and air-dried. Slides were stained with: CD8 (1:200), Ki-67 (1:100), and granzyme B (1:100) (BD Pharmingen). The tissues were blocked with 2% goat serum for 1 h and washed before incubating with the antibody for 1 h at 25 °C. The sections were then washed, mounted with vector shield (Sigma) and viewed under a Zeiss fluorescent microscope.

2.8. Proliferation assays

Polyclonal stimulation of PBMCs from healthy donors and the CD25 deficient patient was performed by culturing at 2×10^5 cells per well in duplicate in flat-bottom 96-well tissue culture plates (Corning) with plate bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of IL-2 low (10 U/ml), medium (100 U/ml), high (1000 U/ml), IL-2 (100 U/ml) + IL-15 (10 ng/ml), or with PHA alone (1 ug/ml). Ag-specific responses were measured using 1×10^5 PBMCs in 96-well round bottom tissue culture plates (Corning) containing the respective Ags. Each antigen (*Candida albicans* strain CA-2) (1.25×10^5 spores per well; kindly provided by Dr. L. Romani Perugia, Italy), Tetanus Toxoid (TT) (5 μg/ml (Enzo)), CMV (2.5 μg/ml Towne strain; kindly provided by Dr. Chiara Bonini, Milan, Italy), Varicella zoster (VZV) rod strain (2.5 μg/ml (Advanced Biotechnology)) and herpes simplex virus (HSV) MacIntyre strain (2.5 μg/ml (Advanced Biotechnology)) were cultured with the cells in the presence or absence of IL-2 (100 U/ml) and IL-15 (10 ng/ml). The cells were cultured for 3–4 days before pulsing with [^{3}H] thymidine for 18 h. Plates were harvested and proliferation was recorded as counts per minute (cpm) using a gamma counter.

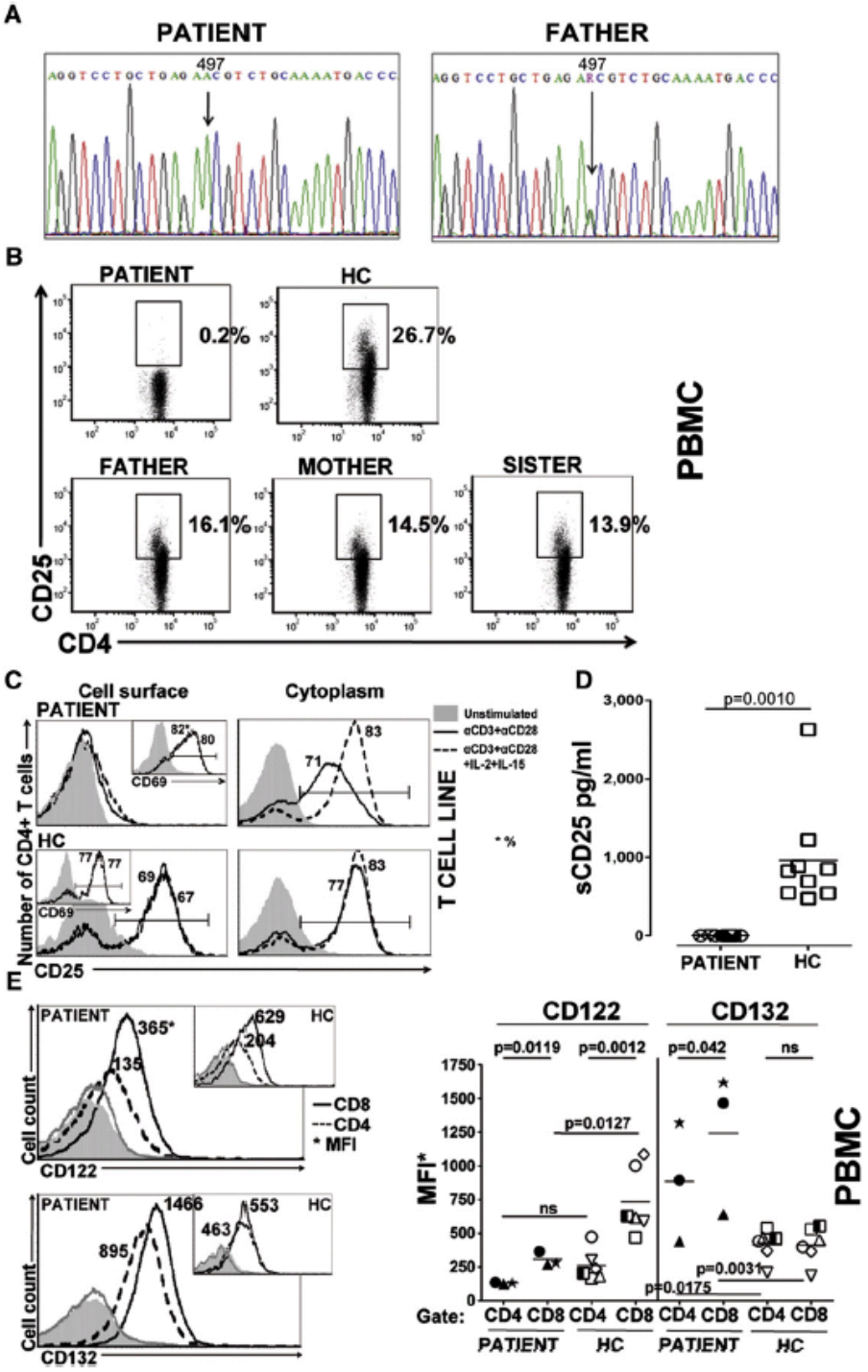
2.9. ELIspot

The frequency of interferon γ (IFNγ) producing cells was determined by ELIspot (BD Pharmingen) as previously described [19]. Briefly, 2×10^5 freshly isolated PBMCs were stimulated in the presence or absence of attenuated CMV (2.5 μg/ml Towne strain), IL-2 (100 U/ml), and or IL-15 (10 ng/ml). After 18–20 h of incubation, plates were washed and developed according to the established protocol. Spots were counted using an ELIspot reader adjusted to eliminate background spots (Aoelovis).

2.10. CMV proliferation

CMV-specific proliferative responses of T cells from the CD25 deficient patient and healthy controls were tested *in vitro*. PBMCs (4×10^6) were harvested from each donor and were labeled in 5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described [20]. The cells were stimulated with live-attenuated CMV (2.5 μg/ml Towne strain), and/or IL-15 (10 ng/ml) for 5 days before harvesting. The resulting cells were stained with anti-CD4 and anti-CD8 antibodies and acquired by flow cytometry. The proliferation was determined by CFSE dilution for the respective populations indicated by decreasing MFI.

Figure. 1 A c.497G>A mutation in the *IL2RA* gene inhibits surface and soluble CD25 expression. A, Sequence trace of genomic DNA of the patient (left panel) showing a G to A substitution at position 497 (down arrow) in exon 4 leading to the amino acid substitution S166N. Sequence trace of paternal genomic DNA shows a single copy of the G497A mutation (right panel). B, Representative dot plots from 3 independent experiments of CD25 expression on $CD4^+$ T cells from PBMCs of the CD25 deficient patient, the patient's father, mother and sister and a healthy control (HC) (n=6) as determined by flow cytometry. C, Representative histograms of surface (left column) and cytoplasmic (right column) expression of CD25 on T cell lines were determined for the patient (bottom histograms) and healthy controls (upper histograms) after stimulation with anti-CD3 and anti-CD28 alone (solid line) or in combination with IL-2 and IL-15 (dotted line) or left untreated (grayed area). Embedded histograms of CD69 expression for the patient and healthy controls are shown. D, Soluble CD25 (sCD25) was measured from the plasma at 8 different time points from the patient and from 9 healthy controls. E, Representative histograms showing the expression of CD122 (upper left) and CD132 (lower left) were determined on PBMCs of the patient (large histograms) and healthy controls (embedded histograms). The MFI of CD122 and CD132 on the $CD4^+$ and $CD8^+$ T cells of the patient (each symbol indicates a unique time point) and healthy controls (n=6) was determined using FACS analysis on PBMCs.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SISTER
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CD25
CD4
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Cell surface
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E
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MFI*
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2.11. Suppression assay

The sensitivity to suppression of $CD8^+$ effector T cells by Tregs was performed using allogeneic Treg cells as suppressors. $CD8^+$ T cells were purified from the CD25 deficient patient, the patient's mother and father and a healthy donor using $CD8^+$ T cell beads (Miltenyi) according to the manufacturer's protocol ($CD8^+$ T cell purity was determined to be >95% for all samples), and were labeled with 5 μM of CFSE. Tregs were isolated from the patient's mother and father and healthy donors using $CD4^+CD25^+$ Treg isolation kit (Miltenyi) to high purity and were seeded in a 96 well U bottom plate in triplicate at a 0.5:1 ratio (1.25×10^4 suppressor cells to 2.5×10^4 responder cells) in complete X-Vivo (Biowhitaker). The cells were then stimulated with inspector beads (Miltenyi) using 2 beads per cell in the presence or absence of IL-2 (100 U/ml) for 5 days. The cells were then harvested and stained for CD8 to determine the level of proliferation in each condition.

2.12. Statistics

Statistical significance of mean differences for each parameter was determined by 2-tailed Student's *t* test or by ANOVA followed by post-hoc Bonferroni test for multiple comparisons where appropriate. Data are expressed as mean±SEM, and a *p* value less than 0.05 was considered significant. All calculations were done using GraphPad Prism software ver.5 (GraphPad Software).

3. Results

3.1. Case report

The patient is an 8 year-old female born to consanguineous parents (first cousins) of Italian descent, who developed symptoms resembling IPEX, such as diffuse eczema and severe diarrhea, during her first month of life. The enteropathy with severe villous atrophy was diagnosed as of autoimmune origin and complicated with CMV infection, which was treated by Gancyclovir, but became recurrent. The patient suffered from enteropathy until 5 years of age, often requiring parenteral nutrition and was treated with continuous combination therapy of steroids and Tacrolimus or Tacrolimus alone.

At 1 year of age, she developed a bullous pemphigoid as a reaction to hexavalent vaccination and was treated with plasmapheresis. She presented an autoimmune thyroiditis at 4 years of age, which was treated with hormone replacement therapy until the thyroiditis resolved at 7 years. She was constantly treated with immunosuppressive therapy over all 6 years, but only with partial and transient benefit. When she was 5 years old, she developed diffuse eczema and alopecia universalis despite the immunosuppressive therapies. The skin lesions became a severe form of psoriasiform dermatitis, which extended to the whole body and was characterized by erythroderma and scaling skin associated with multiple lymphadenopathies of inguinal, axillary, nucal and dorsal lymph nodes, diagnosed as hyper-reactive lymph nodes by ultrasonographic examination. During the last year of her follow-up, genital skin lesions were complicated by a severe cellulitis caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, requiring multiple antibiotic therapy. At 8 years and 6 months of age, due to the evolving dermatitis, Methotrexate was also started with no benefit. Therefore, Mycophenolate Mophetil was given alone with limited improvement of skin lesions, but improvement of the lesions was only seen when combined with rapamycin. Additional therapies included prophylactic treatment, against *Pneumocystis jiroveci* and fungi, and Gancyclovir upon detection of CMV reactivation. Hematopoietic stem cell transplantation was indicated as the only curative treatment for the disease and since the patient did not have a matched family donor, a search for matched unrelated donors from the Registry was initiated.

While the patient was in our hospital between the ages of 8 years and 4 months and 8 years and 9 months, her WBC (8933/μL±3033; normal range: 5500–15,500/μL), and total lymphocyte counts (2821/μL±1194; normal range: 2000–8000/μL) were in the normal range. However, the patient showed an inverted CD4/CD8 ratio (0.8±0.4; normal range: 0.9–2.6) with normal CD4 counts (1000/μL±341; normal range 300–1300/μL) and high CD8 counts (1574/μL±855; normal range 300–1300/μL). In addition, low B (77/μL±52; normal range 200–1600/μL) and NK (83/μL±72; normal range: 90–900/μL) cell counts persisted during her stay. C-reactive protein (CRP) was negative. Serum IgG and IgM were normal (1150 mg/dL — normal range: 633–1016 mg/dL — and 149 mg/dL — normal range: 56–261 mg/dL, respectively), while IgA and IgE were elevated (409 mg/dL — normal range: 41–315 mg/dL and >5000 kUA/L — 1.6–60 kUA/L, respectively). Serum analysis showed the presence of protective titers of IgG anti-Tetanus, anti-Hepatitis B and anti-CMV, while she did not have anti-Herpes Simplex Virus-1 and anti-Herpes Simplex Virus-2 antibodies. The screening for celiac disease was negative. The serum screening for other auto-antibodies (anti-pancreatic insula (ICA), anti-glutamic acid decarboxylase (GAD), anti-insulin, anti-IA2, anti-ZNT8, anti-thyroglobulin, anti-thyroperoxidase, anti-liver kidney microsome (LKM), anti-adrenal glands, ANA, ENA) was negative. The NK activity measured as $CD107a^+$ cells in the presence of IL-2 or IL-15 against K562, was preserved (data not shown).

3.2. Novel *IL2RA* mutation

The molecular analysis of the *IL2RA* gene of the patient's DNA revealed the presence of a previously unreported mutation (Fig. 1A). The patient was homozygous for a c.497G>A transition in exon 4, leading to an amino acid substitution at codon 166 (S166N) of the protein. The patient's father (Fig. 1A), mother, and sister proved to be heterozygous for the same mutation (data not shown).

3.3. CD25, CD122 and CD132 protein expression analysis

Immunophenotype analysis of freshly isolated PBMCs from the patient showed the absence of CD25 expression on the surface of $CD4^+$ T cells (Fig. 1B). On the other hand, CD25 was detected on $CD4^+$ T cells of the parents and the sister of the patient (16%, 14.5%, 13.9% for father, mother, and sister respectively), although at a lower frequency than

the healthy controls (26.7±6.5, n=5), which might be explained by the patient's kin being heterozygous for the mutation (Fig. 1B).

CD25 surface expression on CD4⁺ T cell lines of the patient remained undetectable upon TCR mediated activation even in the presence of exogenous IL-2 and IL-15 (Fig. 1C). Control CD4⁺ T cell lines tested in parallel, showed increased surface CD25 expression. Importantly, TCR activated CD4⁺ T cells of the patient expressed CD25 in the cytoplasm, as well as at the mRNA level (data not shown), in a comparable amount to the healthy control (Fig. 1C). This raised the question whether CD25 was rapidly shed from patient's cell surface or remained intracellular. Soluble CD25 (sCD25) was undetectable in the sera (Fig. 1D) or in cell supernatants of activated PBMCs (Supplementary Fig. 1) from the patient compared to healthy control samples demonstrating that the *IL2RA* mutation of the patient inhibits plasma membrane expression and subsequent shedding of the CD25 protein.

To evaluate whether other IL-2R subunits were affected by the loss of CD25 surface expression, CD122 and CD132 expression was determined on CD4⁺ and CD8⁺ T cells. In healthy controls, CD8⁺ T cells expressed CD122 at higher intensity than CD4⁺ T cells as determined by Mean Florescence Intensity (MFI) (731.7±249.5 and 261.1±121.3, respectively, p=0.0012) but there was no significant difference in CD132 expression between CD8⁺ and CD4⁺ T cells (497.9±270.4 and 495.7±308.9, respectively, n=6) (Fig. 1E). Compared to healthy controls, CD8⁺ T cells of the patient expressed lower levels of CD122 (p=0.0127), while, both CD8⁺ and CD4⁺ T cells of the patient expressed significantly higher levels of CD132

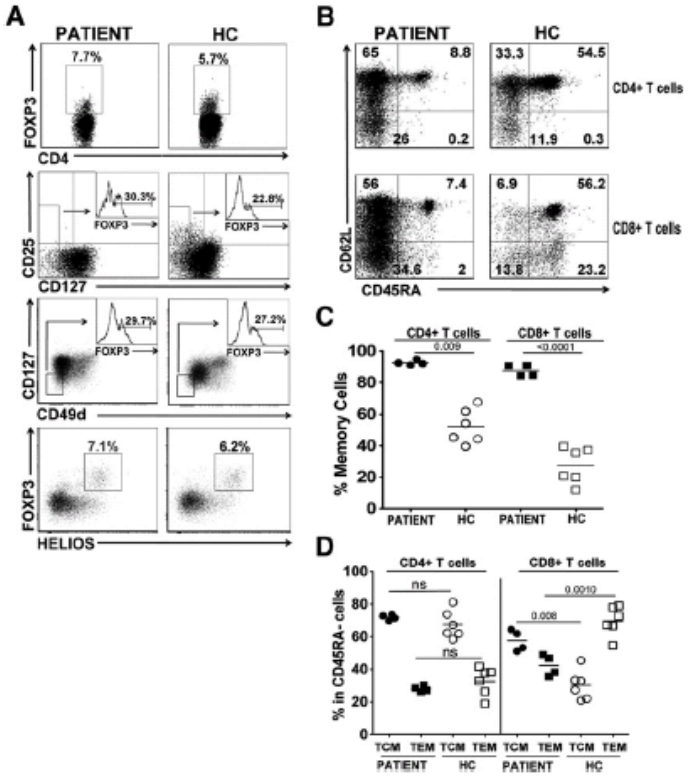


Figure. 2 CD25 deficiency promotes a T memory cell phenotype. A, Representative FACS plots depicting the average percentage of FOXP3⁺ CD4⁺ T cells (upper panels), CD127^{low} cells expressing FOXP3 (middle upper panels with embedded histograms), CD127^{low}CD49d^{low} cells expressing FOXP3 (middle bottom panels with embedded histograms), FOXP3⁺ and HELIOS⁺ staining were determined in the CD25 deficient patient (average of 2–4 different time points is shown) and healthy controls (HC; average percentage of 2–6 different donors is shown). B, The average percentage of naïve (CD45RA⁺CD62L⁺), central memory (TCM, CD45RA⁻CD62L⁺), effector memory (TEM; CD45RA⁻CD62L⁻) for CD4⁺ (upper row) and CD8⁺ (lower row) T cells for the patient (left column) and healthy controls (right columns). The percentages shown are the average of 4 different time points for the patient and the average of six healthy donors. C, The overall percentage of memory cells (CD45RA⁻) from the patient and healthy controls. D, The percentage of the effector populations within the memory pool (CD45A⁻) of the patient and healthy controls.

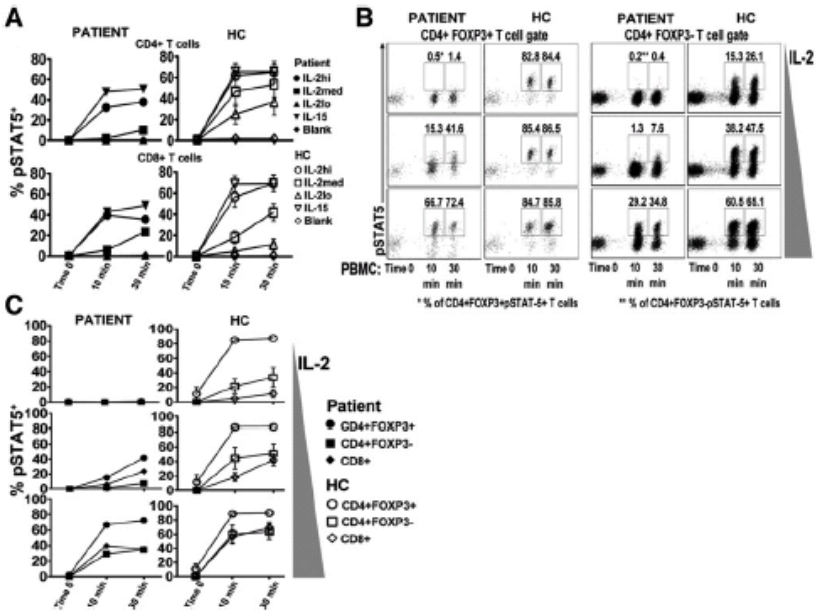


Figure. 3 Reduced IL-2 response of T cells from the CD25 deficient patient but FOXP3$^+$ T cells remain first responders. A, The percentage of CD4$^+$ (upper row) and CD8$^+$ (lower row) T cells from PBMCs of the patient (left column) or healthy controls (right column; n=5) responding to IL-2lo (10 U/ml), IL-2med (100 U/ml), IL-2hi (1000 U/ml), or IL-15 (10 ng/ml) at 0, 10 or 30 min. pSTAT5 was evaluated by flow cytometry from barcoded cells. B, Representative dot plots of the IL-2 responsiveness of CD4$^+$FOXP3$^+$ and CD4$^+$FOXP3$^-$ T cells from the patient and healthy control were evaluated for using IL-2lo (10 U/ml), IL-2med (100 U/ml), IL-2hi (1000 U/ml) conditions at the indicated time points determined by pSTAT5. C, The hierarchy of IL-2 signaling was determined from PBMCs of the patient (left column; closed shapes) or healthy control (right column; open shapes (n=3)) for CD4$^+$FOXP3$^+$, CD4$^+$FOXP3$^-$ and CD8$^+$ T cells in response to different concentrations of IL-2 as used in B.

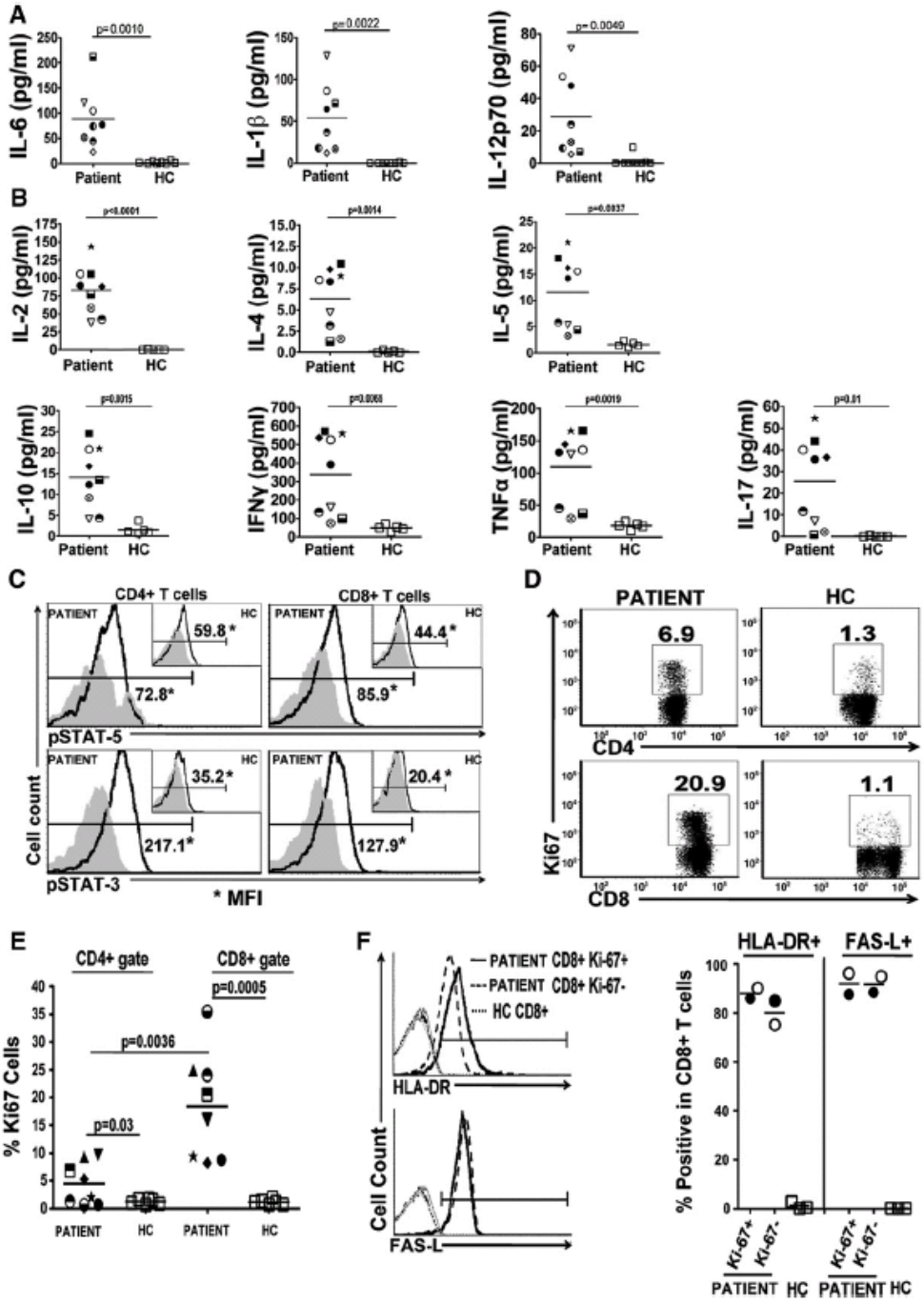
(p=0.0031 and p=0.0175, respectively). These data indicate that, despite the loss of CD25 cell surface expression, both CD122 and CD132 are still expressed on the patient's T cells and CD132 expression is elevated in the patient.

3.4. Lack of CD25 surface expression does not interfere with Treg development and is associated with predominant peripheral memory T cell phenotype

In the patient, the percentage of FOXP3-expressing CD4$^+$ T cells was 7.7±5% (n=4 time points), which was comparable to those of healthy controls (5.7±1.6%, n=19, 7 months to 10 years) (Fig. 2A). This percentage did not seem to change with the stage of the disease. However, the highest percentage of CD4$^+$FOXP3$^+$ T cells (14.4%) was detected within three weeks of rapamycin treatment. We further characterized the Tregs using other Treg-specific markers (Fig. 2A). FOXP3 expressing cells were 30.3±12.2 (n=4 time points) in CD4$^+$CD127^{low} gate for the patient, which were comparable to healthy control values (22.8±13.6%, n=5). Additionally, FOXP3-expressing T cells of the patient in CD4$^+$CD49d^{low}CD127^{low} gate ranged between 12.5 and 62.1% (mean: 29.7%, n=4 time points), which was similar to healthy control values with variability (22.2±6.5%, n=5). Similarly, the proportion of CD4$^+$ T cells co-expressing HELIOS^{hi} and FOXP3$^+$ showed no disparities with healthy

Figure. 4 Abundant innate and adaptive cytokines *in vivo* correlate with predominate CD8$^+$ activation and proliferation. Sera from the CD25 deficient patient (unique symbol for each time point) and healthy donors (each symbol represents a unique patient) were measured for innate (A) and adaptive (B) associated cytokines. C, Representative FACS plots of pSTAT5 (upper row; bold line) and pSTAT3 (lower row; bold line) in CD4$^+$ and CD8$^+$ T cells of the CD25 deficient patient (larger histogram) and healthy controls (embedded histogram) using isotype control antibody staining (grayed area) as a reference. D, Proliferating CD4$^+$ and CD8$^+$ T cells from fresh PBMCs were determined by staining for Ki-67$^+$ (representative FACS plot shown). The average proliferative rate of CD4$^+$ and CD8$^+$ T cells from the CD25 deficient patient and healthy controls (E) was taken at different time points and averaged (p value; students *t* test). F, Representative histograms of HLA-DR and FAS-L expression on proliferating (Ki-67$^+$; solid line) and non proliferating (Ki-67$^-$; dashed line) CD8$^+$ T cells from the CD25 deficient patient at two different time points or total CD8$^+$ T cells from healthy controls (dotted lines). Gray line is the staining control on the CD8$^+$ T cells.

controls (7.1±4% (n=2 time points) and 6.2±4.5% n=2 respectively), and the expression of CTLA4 and GITR on FOXP3$^+$ T cells was found at comparable levels (data not shown). The presence of bona fide Tregs was confirmed at the molecular level by the analysis of the T-regulatory cell-specific demethylated region (TSDR) of *FOXP3* [21]. To elucidate the relative proportion of Tregs in the CD3$^+$ T cell population, we normalized for the percentage of total



T-lymphocyte-specific demethylated region (TLSDR) of *CD3* [22]. The patient displayed 3.80% of Tregs in $CD3^+$ cell population, thus under the 25° percentile (<4.21%) of the normal range (normal values 1.8–8.1%) [23], even if the percentage of Tregs determined in purified $CD4^+$ T cells by TSDR demethylation analysis was 13.8%, a value slightly above those of healthy controls (6.5–12.2%, median: 7.5%, n=7, 3 months to 13 years). Overall these results indicated that peripheral Tregs were present in the patient despite the absence of a functional CD25, albeit at an overall reduced level in total lymphocytes.

Further characterization of peripheral T cells showed increased $CD45RA^-$ memory $CD4^+$ (92.5±1.6%) and $CD8^+$ (87.5±3.6) T cells in the patient (n=4 time points; Figs. 2B and C) compared to adult normal donors and to memory T cell values for age ($CD4^+$ T cell memory: 24.0–43.4%; $CD8^+$ T cell memory: 13.4–29.9%, [24]). Within the increased $CD4^+$ memory T cell populations, $CD45RA^-CD62L^+$ central memory (TCM) cells were predominant over $CD45RA^-CD62L^-$ effector memory (TEM) phenotype (Fig. 2D). $CD4^+$ TCM and TEM cells of the patient showed a distribution pattern similar to those of healthy controls; however, the patient had a greater proportion of $CD8^+$ TCM cells in $CD8^+$ in the memory T cell population. These results indicated that *IL2RA* mutation in the patient led to predominant memory phenotype of both peripheral $CD4^+$ and $CD8^+$ T cells, with significant increase in $CD8^+$TCM cells proportionally.

3.5. High doses of exogenous IL-2 are required to induce STAT5 signaling in CD25 null T cells while $FOXP3^+CD4^+$ Tregs remain the first responders

CD25 is a fundamental part of the high affinity IL-2R complex. To demonstrate that CD25 surface expression confers the ability of $CD4^+$ T cells to respond rapidly to IL-2, phosphorylated (p) STAT5 was determined in CD4 T cells expressing different levels of CD25 in healthy donors: CD25 high (CD25hi), $CD25^+FOXP3^+$(which includes Tregs), CD25 medium (CD25med) and CD25 negative ($CD25^-$) (Supplementary Fig. 2). The level of pSTAT5 was consistent with the intensity of CD25 expression. In the presence of IL-2 at low concentrations, $CD4^+CD25^{hi}$ and $CD4^+CD25^+FOXP3^+$ T cells had the highest percentage of $pSTAT5^+$ cells while $CD4^+CD25^{med}$ T cells were intermediate responders and $CD4^+CD25^-$ responded poorly. Responses to increasing levels of IL-2 concentrations did not alter the sensitivity of each population to IL-2. Thus, this data demonstrates that high expression of CD25, including in $FOXP3^+$ Treg, equips $CD4^+$ T cells to respond preferentially to low levels of IL-2 and have a higher potential to signal through STAT5.

Since the patient's cells did not express CD25 on their surface, signaling through the intermediate affinity IL-2R (CD122 and CD132) was questioned. T cells from the CD25 null patient were tested for their ability to induce pSTAT5 in response to IL-15 and IL-2 (Fig. 3A). The patient's $CD4^+$ and $CD8^+$ T cells displayed a pSTAT5 pattern similar to healthy controls (n=3) in the presence of IL-15, but with a slight decrease at all time points (10 and 30 min). These data demonstrate that in the absence of cell surface CD25, CD122 and CD132 are functional in the patient's T cells. To the contrary, $CD4^+$ T cells of the patient had dramatically reduced pSTAT5 at all concentrations of IL-2 tested. $CD4^+$ T cells of healthy controls (n=3) induced pSTAT5 at all time points with IL-2 stimulation in a time and dose dependent manner, while the patient's $CD4^+$ T cells responded only to the high concentration of IL-2. Overall, the percentages of $pSTAT5^+$ $CD4^+$ T cells from the patient, even stimulated at high concentrations of IL-2, were lower than those of healthy controls (n=3), indicating an overall defect in IL-2 signaling in $CD4^+$ T cells. $CD8^+$ T cells of the patient were less affected by the loss of CD25 in response to IL-2 stimulation. The pSTAT5 levels in the patient's $CD8^+$ T cells with high concentration of IL-2 were similar to the percentages of $CD8^+$ T cells of healthy controls stimulated with high and medium concentrations. Together, these data show that $CD4^+$ T cells from CD25 null patients are more affected than $CD8^+$ T cells in response to IL-2 when CD25 surface expression is absent. The alteration in responsiveness results in the patient's $CD8^+$ T cells becoming more reactive to IL-2 than $CD4^+$ T cells at the medium doses tested.

The comparison of STAT5 signaling in $CD4^+FOXP3^+$ and $FOXP3^-$ T cells of the CD25 null patient *versus* normal donors shows that neither $CD4^+FOXP3^+$ nor $CD4^+FOXP3^-$ T cells of patient responded to low concentration of IL-2 (Fig. 3B). Both $CD4^+FOXP3^+$ and $CD4^+FOXP3^-$ T cells had pSTAT5 detection with medium and high concentration of IL-2 in a time and dose dependent manner (Fig. 3B). However, pSTAT5 levels in the patient's cells never reached those of healthy controls. Importantly, as seen in healthy controls, pSTAT5 response in $CD4^+FOXP3^+$ T cells was always higher than $CD4^+FOXP3^-$ T cells. This indicated that even though $CD4^+FOXP3^+$ T cells lacked CD25 expression, they were able to transmit IL-2 signaling with medium and high IL-2 stimulation. Therefore, they were still the first $CD4^+$ T cell subtype responding to IL-2, and their pSTAT5 status was always higher than $CD4^+FOXP3^-$ T cells.

The IL-2 signaling hierarchy of $CD4^+FOXP3^+$, $CD4^+FOXP3^-$ and CD8 T cells was also evaluated. In the CD25 null patient and in healthy donors, $CD4^+FOXP3^+$ T cells were always the highest responders to IL-2 in all conditions (Fig. 3C). The percentage of pSTAT5 in CD25 null $CD8^+$ T cells was always higher than $CD4^+FOXP3^-$ T cells in intermediate and high concentrations of IL-2, whereas the $CD8^+$ T cells of healthy donors had significant levels of pSTAT5 only in the presence of high doses of IL-2 (Fig. 3C). This data indicates that in the absence of CD25 surface expression 1) the threshold required for IL-2 signaling in all T cell subsets is raised, 2) $CD4^+FOXP3^+$ are the first to respond to IL-2 despite the absence of CD25, and 3) unlike in healthy donors, $CD8^+$ T cells from CD25 null patients preferentially respond to IL-2 compared to $CD4^+FOXP3^-$.

3.6. High serum cytokine levels associate with pSTAT5 and pSTAT3, hyperproliferation, and activation of CD25 null T lymphocytes *in vivo*

To determine why the CD25 null patient had high circulating T lymphocytes, cytokine levels were evaluated in serum. All cytokines tested, innate immune (IL-6, IL-1β, IL-12p70) (Fig. 4A) and Th1 (IL-2, IFNγ), Th2 (IL-4, IL-5, IL-10), Th17 (IL-17), and TNFα (Fig. 4B) were above that of age-matched control, tested over a period of nine months in the presence

of varying levels of immunosuppressive drugs. This data demonstrates that the production of all cytokines tested was not impaired despite the absence of CD25 surface expression, suggesting that high cytokine levels found in circulation may sustain proliferation *in vivo*. To determine if CD25 null T cells are activated by the cytokines *in vivo*, freshly isolated peripheral $CD4^+$ and $CD8^+$ T cells of the patient were first evaluated for active STAT5 and STAT3 signaling (Fig. 4C). pSTAT5 levels in $CD4^+$ T cells (72.8 MFI) and in $CD8^+$ T cells (85.9 MFI) of CD25 null patient (59.8 MFI and 49.8 MFI, n=2 time points) were higher than in healthy controls (44.4 MFI and 47.3 MFI, n=2). Similarly, both $CD4^+$ and $CD8^+$ T cells possessed higher levels of pSTAT3 (217.1 MFI and 127.9 MFI respectively) than healthy controls (35.2 MFI and 77.4 MFI, n=2; 20.4 MFI and 45.4 MFI, n=2, respectively). This data indicates that cytokine signaling pathways in circulating T lymphocytes of the CD25 null patient had active *in vivo* signaling to both STAT3 and STAT5 pathways.

Additionally, we found that peripheral $CD8^+$ and $CD4^+$ T cells of the patient were highly proliferative *in vivo* as evident by the expression of Ki-67 (Figs. 4D–E). Ki-67 expression ranged between 8.2 and 35.5% (n=8) in $CD8^+$ T cells which was significantly higher than healthy control levels (1.22±0.45%, n=6) at all different time points evaluated (p=0.0005). $CD4^+$ T cells did not always express Ki-67 at high levels (0.7–9.7%, n=8), but overall Ki-67 expression was increased compared to healthy control values (1.28±0.43%, n=6), (p=0.03). Importantly, an increase in Ki-67 expression was more prominent in $CD8^+$ T cells than $CD4^+$ T cells for all time points detected (p=0.0036). Although Ki-67 expression remained high in $CD8^+$ T cells the lowest point was during rapamycin treatment. Both proliferating and non-proliferating $CD8^+$ T cells had high surface expression of HLA-DR (86% and 90.1%, n=2; 85% and 75.2%, n=2, respectively) and FAS-L on the cell surface consistent with the highly activated phenotype of $CD8^+$ T cells (87.6% and 96.3%, n=2; 88.4% and 95%, n=2, respectively) (Fig. 4F). However, BCL-2 expression, an anti-apoptotic protein, was low in proliferating $CD8^+$ T cells (40.7% and 52.1%, n=2) but comparable to healthy donors in non-proliferating cells (data not shown). Irrespective of the BCL-2 levels, T cells from the CD25 deficient patient were not prone to apoptosis compared to healthy donors (data not shown). Decreased BCL-2 expression in proliferating peripheral $CD8^+$ T cells and increased FAS-L expression in highly activated $CD8^+$ T cells suggest that intrinsic and extrinsic apoptotic pathways in $CD8^+$ T cells were not disrupted in the patient carrying CD25 mutation.

3.7. Proliferating $CD8^+$ T cells mediate autoaggression in skin

The morphological analysis of skin biopsy of the patient's, epidermis showed hyperplasia with hyper-orthokeratosis (Fig. 5A). A dense small to medium lymphocytic infiltrate was observed mainly in upper dermis (Fig. 5B). Infiltrating lymphocytes almost exclusively displayed a T cell phenotype ($CD3^+$) (Fig. 5C), while B-cells ($CD20^+$) were occasionally encountered, and staining for immunoglobulins was negative (data not shown). T cells were almost invariably $CD8^+$ (Figs. 5D–F). Many of these skin infiltrating $CD8^+$ T cells were proliferating as determined by double immunofluorescence staining with Ki-67 (Fig. 5E). Additionally, they were granzyme B^+ ($GrzB^+$) $CD8^+$ T cells (Fig. 5F). TCR rearrangement assessed by PCR both on skin biopsy and peripheral blood revealed a polyclonal T-cell population (data not shown). These data show that $CD8^+$ T cells highly infiltrate the skin, undergo proliferation, and present lytic capacity, suggesting that $CD8^+$ T cells are mediating skin aggression manifesting as severe erythrodermia. However, the $CD8^+$ T cells display normal sensitivity to suppression when co-cultured with Tregs from normal donors (Supplementary Fig. 3).

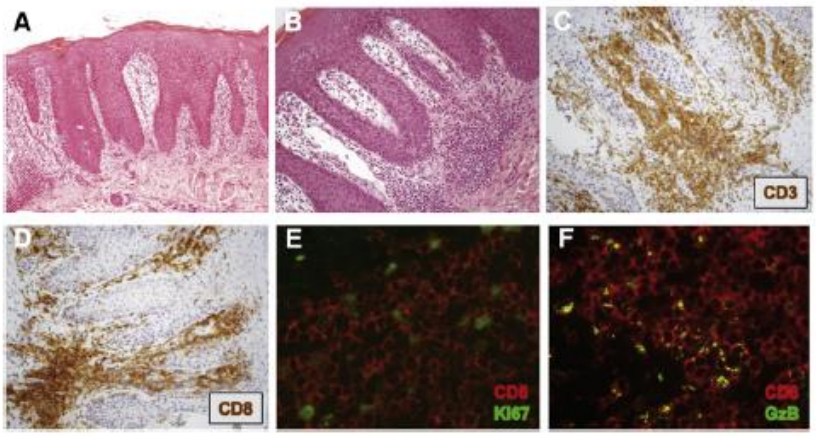


Figure. 5 Epidermal hyperplasia due to $CD8^+$ T cell infiltration, proliferation and granzyme B production. A, A skin biopsy of the patient showed hyperplasia with hyper-orthokeratosis. (B), Lymphocytic infiltrate was observed mainly in upper dermis, (C) displaying mostly a T cell phenotype ($CD3^+$). Skin infiltrating cells were mostly $CD8^+$ T cells (D–F) and many were proliferating as determined by double immunofluorescence staining with CD8 and Ki-67 (E). F, A high frequency of $CD8^+$ T cells was also granzyme B^+.

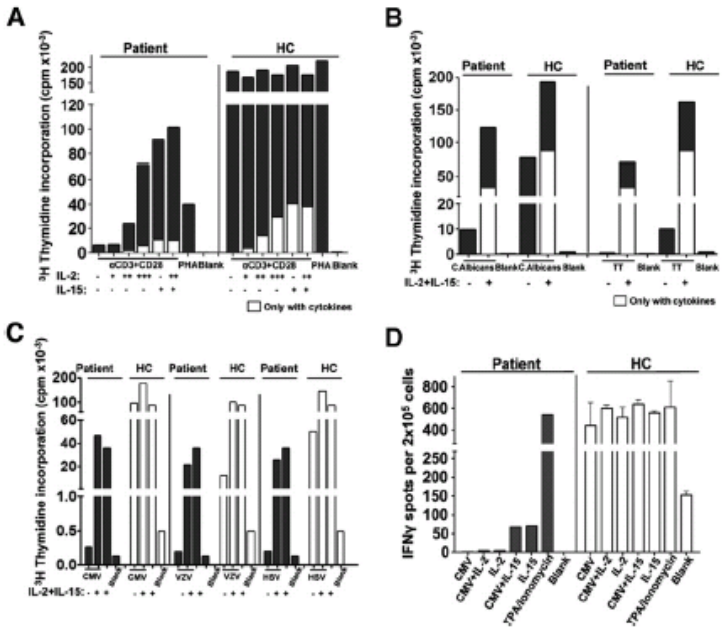


Figure. 6 Defective T cell responses to polyclonal mitogens and viral Ags. A, PBMC from the patient and healthy controls were stimulated with anti-CD3 and anti-CD28 in the presence or absence of IL-2 (+ 10 U/ml, ++ 100 U/ml, and +++ 1000 U/ml) and/or IL-15 (10 ng/ml) and/or IL-15 (10 ng/ml), or PHA and proliferation was determined by ^{3}H-thymidine after 3 days. B, T cell responses to *C. albicans*, TT, were measured after 4 days and (C) different viruses (CMV, VZV, and HSV) were measured after 3 days of stimulation in the presence or absence of IL-2 and IL-15. D, PBMC ELIspot assays from the patient and healthy control were stimulated with CMV Ags alone or in the presence of IL-2 or IL-15, or with IL-2, IL-15, or TPA/Ionomycin alone.

3.8. *In vitro* proliferation defect to polyclonal mitogens was rescued with exogenous IL-2, but viral-specific response was consistently absent

One of the main clinical manifestations of the patient, other than autoimmunity, was chronic viral infections especially CMV. We tested the ability of the patient's PBMCs to respond to Ag-specific and non-specific TCR activation. The patient's PBMCs had poor *in vitro* proliferation response to TCR-mediated activation compared to healthy controls (Fig. 6A), which was partially rescued by exogenous IL-2 and IL-15. T cell responses to *C. albicans*, Tetanus Toxoid (TT), and different viruses (CMV, VZV, and HSV) were also impaired (Figs. 6B–C). Similarly, the patient's PBMCs did not produce IFN-γ upon CMV activation, as determined by ELISPOT assay. The addition of high concentrations of IL-2 or IL-15 did not rescue IFN-γ production (Fig. 6D) or CD4 or CD8 CMV-specific proliferation (Supplementary Fig. 4). These results demonstrate that T cells from the CD25 null patient poorly respond to polyclonal mitogens and to microbial and viral Ags despite the persistent *in vivo* exposure to CMV.

4. Discussion

Studies of primary immunodeficiencies have been fundamental in defining the function(s) of specific molecules in health and disease. The present work represents a detailed study on the immunological status of a patient lacking CD25 surface expression due to a novel point mutation. The chronic effect of this mutation led to the development of progressive manifestations of both autoimmunity, such as enteropathy, erythrodermia and severe alopecia, and immunodeficiency with chronic CMV infection. Profound alterations of the peripheral T cell subsets consisted of a complete skew towards increased CD8$^+$ T cells over CD4$^+$ T cells, expansion of the memory T cell compartments, with preservation of FOXP3$^+$ T regulatory cells, whereas B cells and NK cells were persistently low. Increased expression of T cell activation markers and high serum levels of innate and adaptive cytokines were detected in parallel with high *in vivo* proliferation of T cells. Despite the lack of surface CD25, both Tregs and T effector cells remained able to respond to cytokines, although impaired in their sensitivity to IL-2, as demonstrated by pSTAT5. Among the peripheral T effector cells, CD8$^+$ T cells of the CD25 deficient patient highly expressed CD132, and this increased expression made them more responsive to IL-2 than CD4$^+$ T effector cells. Proliferating CD8$^+$ GrzB$^+$ T cells aggressively infiltrated the skin, suggesting that they were the main mediators of the tissue damage. However, Ag-specific T cell responses were deeply impaired *in vitro* and *in vivo*. Therefore, these data demonstrate that the persistent lack of CD25 surface expression hinders the development of effective Ag-specific T cell responses while cytokine

driven polyclonal T cell proliferation and activation mediate tissue damage.

Similar to the reported CD25 deficient cases, CMV infection and severe early onset enteropathy were the first signs of the deficiency in the patient studied [14,16]. The failure to resolve CMV infection in CD25 deficient patients and other primary immunodeficiencies, could logically explain a skewed CD4/CD8 T cell ratio. Since CMV infection induces innate immune responses and leads to the expansion of CMV-specific effector T cells, one would predict that the expansion of the $CD8^+$ T cell compartment in CD25 deficient patients is due to the response to CMV Ags [25]. Surprisingly, the patient's T cells responded poorly to viral, bacterial and fungal Ags. T cell exhaustion and increased sensitivity to apoptosis were ruled out as possible causes for decreased Ag-specific responsiveness (data not shown). The impairment of effector functions by the $CD8^+$ T cells could be due to the lack of CD25 during priming. Bevan's group demonstrated in mouse that CD25 expression during a primary immune response is critical for programming protective memory cells to respond to Ag during re-exposure [26]. Thus, the absence of surface CD25 expression in the patient presumably impairs the induction of effective Ag-specific memory T cell responses thus explaining the patient's inability to clear infections. In addition, the reported dysfunction of CD25 deficient dendritic cells could contribute to ineffective Ag presentation and T cell activation [27]. It is tempting to speculate that the persistent infections (*i.e.* CMV) and the innate immune responses thereof, act in concert to promote non-specific $CD8^+$ T cell proliferation probably as a result of the accumulation of the numerous cytokines like the ones found in the serum of the patient. Indeed, based upon our findings, CD25 deficiency altered the hierarchical signaling in response to IL-2 in favor of $CD8^+$ T cells over the $CD4^+$ T cells, contrary to what is observed in healthy subjects. This switch in IL-2 sensitivity together with increased IL-2 levels in serum could be the driving force in the expansion of $CD8^+$ T cells in the absence of cell surface CD25 expression. Supporting this notion are studies performed in the CD25 KO mouse model that showed that the progressive expansion of memory $CD8^+$ T cells is IL-2 driven [28,29].

Treg cells are clearly present in CD25 deficient patients. In line with the Verbsky study showing the presence of $CD4^+FOXP3^+$ cells [16], we detected a normal frequency of $CD4^+FOXP3^+$ Tregs that also had a similar phenotype to Tregs from normal donors ($CD127^{LO}CD49d^-HELIOS^+CTLA4^+GITR^+$). However, we demonstrate that the proportion of bona fide Tregs with demethylated TSDR was low when normalized to the whole lymphocyte compartment. These findings are consistent with the possibility that CD25 is not necessary for $FOXP3^+$ Treg development in humans, but suggest that in the absence of CD25, Tregs remain quantitatively insufficient for an appropriate regulation. Interestingly, CD25 null $FOXP3^+$ Tregs from the patient were still the first to respond to IL-2 (*in vitro*) albeit at higher concentrations than required by healthy subject Tregs. Likely, the most affected function due to CD25 deficiency by $FOXP3^+$ Tregs is the consumption of IL-2. Indeed, upon adoptive transfer of $CD25^+$ $FoxP3^+$ Tregs into CD25 knockout mice, IL-2 levels were dramatically reduced, normal $CD4^+$ and $CD8^+$ T cell ratios were restored and the memory phenotype of both $CD4^+$ and $CD8^+$ T cells returned to near normal levels [28]. Together this indicates that IL-2 consumption by $FOXP3^+$ Tregs is a critical event to maintain the homeostasis of the immune system, and the loss of CD25 surface expression by $FOXP3^+$ Tregs in CD25 deficient patients is likely a contributing factor in the preferential $CD8^+$ T cell proliferation, which are the mediators of autoimmunity. Whether the tissue aggression by $CD8^+$ T cells is auto-Ag driven remains to be clarified. However, the observation that the TCR repertoire of the proliferating T cells is polyclonal argues against it.

Even if CD25 deficiency is considered similar to IPEX syndrome, we clearly highlight major differences in the pathogenesis of the two diseases. Indeed, in IPEX lymphoproliferation involves mainly $CD4^+$ T cells and it is mainly due to loss of regulation by intrinsically dysfunctional FOXP3mut Treg rather than being cytokine driven [30,31]. In the serum of IPEX patients, cytokines are not particularly elevated and Th2 rather than Th1 cytokines are detectable together with increased IL-17 and IL-22, directly related to autoimmunity [32]. In addition, effective immune response to pathogens is preserved in IPEX in which infections mostly occur as secondary to poor clinical conditions or drug dependent immunosuppression. Similarly, autoAb are not readily detectable in CD25 null patients. We therefore demonstrated that even though similarities exist, the pathophysiology of autoimmunity in CD25 deficiency is mechanistically distinct from that of IPEX syndrome, and also from that observed in other monogenic primary immunodeficiencies with autoimmunity, such as Omenn's syndrome, Wiskott–Aldrich or Autoimmune Lymphoproliferative syndrome (ALPS) due respectively to altered thymic deletion of autoreactive T cells, impaired cytoskeleton re-organization during activation of multiple cell type including Tregs [33,34] or defective apoptosis [35].

5. Conclusion

In conclusion, we show that CD25 expression is required to maintain immune homeostasis, and CD25 deficiency is a distinct immunological disease that leads to both an autoimmune and immunodeficiency syndrome that clinically resembles IPEX syndrome. Furthermore, CD25 expression is necessary in human T cells in order to establish a signaling paradigm where IL-2 is utilized in an ordered fashion, and alterations to the hierarchical signaling events can lead to the loss of immune homeostasis. The proposed concept that the failure to properly consume IL-2 and lack of optimal IL-2 signaling contributes to the development of autoimmunity in CD25 deficient patients, could be extended to different autoimmune pathologies in which a reduced CD25 expression occurs as the result of single nucleotide genetic variants of CD25 or of other molecules interfering with proper CD25 signaling. More in depth studies aimed at clarifying this possibility are desirable.

Conflict of interest statement

All authors listed have no conflict of interest regarding this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2013.01.004.

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RESEARCH ARTICLE Open Access

Intergenerational and intrafamilial phenotypic variability in 22q11.2 Deletion syndrome subjects

Emilia Cirillo[1], Giuliana Giardino[1], Vera Gallo[1], Pamela Puliafito[2], Chiara Azzari[3], Rosa Bacchetta[4], Fabio Cardinale[5], Maria Pia Cicalese[6], Rita Consolini[7], Silvana Martino[8], Baldassarre Martire[9], Cristina Molinatto[8], Alessandro Plebani[10], Gioacchino Scarano[11], Annarosa Soresina[10], Caterina Cancrini[2], Paolo Rossi[2], Maria Cristina Digilio[12†] and Claudio Pignata[1,13*†]

Abstract

Background: 22q11.2 deletion syndrome (22q11.2DS) is a common microdeletion syndrome, which occurs in approximately 1:4000 births. Familial autosomal dominant recurrence of the syndrome is detected in about 8-28% of the cases. Aim of this study is to evaluate the intergenerational and intrafamilial phenotypic variability in a cohort of familial cases carrying a 22q11.2 deletion.

Methods: Thirty-two 22q11.2DS subjects among 26 families were enrolled.

Results: Second generation subjects showed a significantly higher number of features than their transmitting parents (212 vs 129, $P = 0.0015$). Congenital heart defect, calcium-phosphorus metabolism abnormalities, developmental and speech delay were more represented in the second generation ($P < 0.05$). Ocular disorders were more frequent in the parent group. No significant difference was observed for the other clinical variables. Intrafamilial phenotypic heterogeneity was identified in the pedigrees. In 23/32 families, a higher number of features were found in individuals from the second generation and a more severe phenotype was observed in almost all of them, indicating the worsening of the phenotype over generations. Both genetic and epigenetic mechanisms may be involved in the phenotypic variability.

Conclusions: Second generation subjects showed a more complex phenotype in comparison to those from the first generation. Both ascertainment bias related to patient selection or to the low rate of reproductive fitness of adults with a more severe phenotype, and several not well defined molecular mechanism, could explain intergenerational and intrafamilial phenotypic variability in this syndrome.

Keywords: 22q11.2 deletion syndrome, DiGeorge syndrome, Immunodeficiency, Phenotypic variability

Background

Chromosome 22q11.2 deletion syndrome (22q11.2DS), also known as DiGeorge or velocardiofacial syndrome (OMIM#188400), occurs in approximately 1:4000 live births [1,2]. Major clinical features include facial anomalies, conotruncal cardiac defects, palatal anomalies, neonatal hypocalcaemia, mild to moderate immune deficiency related to thymic a/hypoplasia [3-5], developmental and speech delay [6]. Ocular, renal and skeletal anomalies may also be found. However, the syndrome has a very wide spectrum of phenotypic features [7,8]. Evidence highlighted that subjects carrying the deletion may have only mild phenotypes [9,10]. Psychiatric or autoimmune disorders [11] can be the features leading to the diagnosis in adolescents and adults, and, in particular, among adults, they may be the unique clinical feature [12,13]. The identification of subjects with attenuated phenotypes is leading to the understanding that the syndrome is more frequent than previously thought and to focus on novel atypical presentations. A wide clinical variability has also been reported even within the same family [9,10,14,15] and a phenotypic discordance has been described among monozygotic twins [16,17]. Genetic modifiers, chance association or environmental interactions have been proposed

* Correspondence: pignata@unina.it
†Equal contributors
[1]Department of Translational Medicine, "Federico II" University, Naples, Italy
[13]Department of Translational Medical Sciences, Unit of Pediatric Immunology, "Federico II" University, via S. Pansini, 5-80131 Naples, Italy
Full list of author information is available at the end of the article

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to explain the intrafamilial variability. Somatic mosaicism or post zygotic second hit have also been hypothesized as potential mechanisms underlying such phenotypic discordance, even though, to date, no definitive explanation is available.

The deletion results from non allelic homologous recombination, occurring during meiosis and mediated by low-copy repeats (LCR) on chromosome 22 [18-20]. Most patients have a deletion of the same 3 Mb region on 22q11.2, including about 30 genes, whereas in 8% of the cases a smaller deletion of 1.5 Mb, which contains 24 genes, is found. So far, no correlation between the severity of the phenotype and the different size of the deletion has been documented [21]. Both deletions include the *TBX1* gene, a member of the T-box family genes. Mice, haploinsufficient for *TBX1*, share several features with humans carrying the homologous deletion, and, in particular, structural cardiac anomalies [22]. Interestingly, both gain or loss of function mutations in *TBX1* have been reported in human subjects exhibiting a DiGeorge-like phenotype [23].

In most cases, the deletion is a sporadic event, while in 8-28% of the cases the syndrome is inherited in an autosomal dominant fashion [8,10,24-26].

Several studies have analyzed the phenotypic variability of the syndrome, but an extensive and conclusive intergenerational and intrafamilial comparison has not yet been reported.

The aim of this study is to perform an intergenerational and intrafamilial comparison of the clinical phenotype in a cohort of patients affected with inherited chromosome 22q11.2DS.

Methods

Patients

Thirty-two subjects (18 females) affected with familial 22q11.2DS from 26 families, were enrolled into the study. The study and data collection, approved by the local Ethics Committee for Biomedical activities "Carlo Romano", have been performed upon informed consent and in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/index.html). Within the group, 17 subjects were from the Italian Network for Primary Immunodeficiencies (IPINET) Registry, followed at 8 Italian Centers, and 15 were referred to two Genetic Units. In 4 families, 2 affected siblings were diagnosed and in one further family, 3 subjects were identified. Mean age +/− SD was 10.4 +/− 7.23 years (range 4 months-31 years). The parent carrying the deletion was the mother in 17 cases (65%) and the father in 9 (35%). Mean age +/− SD of carrier parents was 39.8 +/− 7.9 years (range 21–58 years). We found a preferential maternal transmission, in keeping with the recent observation that female sex represents a significant positive predictor of fitness. All the parents were identified as affected by 22q11.2DS after the birth of a child with the deletion. All patients, but two, were Caucasian. Demographical features are reported in Additional file 1: Table S1. Clinical data of the second generation subjects were obtained, upon informed consent, through the IPINET Registry or from the referring Units. Data on parents carrying the deletion were collected at each Center. Each subject underwent a clinical and laboratory evaluation protocol (IPINET protocol for 22q11.2DS) available at the site http://www.aieop.org. The protocol included cardiologist examination, echocardiography and abdominal ultrasound, which were performed in order to exclude subclinical cardiac or abdominal defects in asymptomatic subjects. To exclude thyroid and calcium-phosphorus abnormalities, the serum levels of calcium, phosphorus, parathyroid hormone, TSH and FT4 were evaluated. History of speech therapy or speech pathologist interventions were recorded for the evaluation of language disorders. The Wechsler Intelligence Scale for Children (WISC) and the Wechsler Adult Intelligence Scale (WAIS) were used for the assessment of cognitive function in subjects of the second generation and in their parents, respectively. Each Center reported the presence/absence of intellectual disability which was defined as an IQ under 70. Neuropsychiatric evaluation was performed by skilled clinicians using the Schedule For Affective Disorders and Schizophrenia for School-aged Children, Present and Lifetime (K-SADS-PL). The second generation subjects, older than 18 years, and their parents were interviewed with the Structured Clinical Interview for Axis I DSM IV Disorders (SCID).

Among the clinical features, birth defects, facial anomalies, gastrointestinal disorders, infections and autoimmune manifestations were recorded. History of neonatal hypocalcaemia was also considered. In 6 families, data were also obtained from the non-deleted parent, to exclude potential interfering factors not related to the 22q11.2 deletion.

Intrafamilial phenotypic variability was assessed through the evaluation of the clinical phenotype in each parent–child couple. Since in 5 families more than 1 subject with the inherited deletion was detected, the phenotype was analyzed in a total number of 32 parent–child pairs from the 26 families.

Cytogenetic analysis

The diagnosis of 22q11.2DS was performed by fluorescence *in situ* hybridization (FISH) analysis and/or multiplex ligation-dependent probe amplification (MLPA) using probes for 22q11 region in all affected patients. In one case, the diagnosis was obtained through a CGH array with whole-genome oligonucleotide microarray Agilent Technologies (Santa Clara, CA) according to the manufacturer protocol.

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Statistical analysis

Statistical analysis was performed using the Student"s t test or the Fisher exact Test. Values of $P < 0.05$ were considered statistically significant. The calculations were performed using InStat software.

Results

Intergenerational clinical phenotypic comparison

Seventeen clinical variables were evaluated in subjects of the second generation and their parents (Table 1), for a total number of 544 and 442 variables in the first and second group, respectively. Overall, affected subjects of the second generation showed a significantly higher number of features than their parents (212 vs 129, $P = 0.0015$). In particular, congenital heart defect (CHD) (62.5 vs 7%, $P < 0.0001$), developmental delay (71.8 vs 42.3%, $P = 0.032$), speech delay (75 vs 46.1%, $P = 0.031$) and calcium-phosphorus abnormalities (37.5 vs 3.8%, $P = 0.0033$) were more represented in the second generation. Conversely, ocular disorders were more frequent in the parents than in their affected children (3.1 vs 23%, $P = 0.037$). Psychiatric (12.5 vs 34.6%), autoimmune (12.5 vs 19.2%) and dental disorders (25 vs 38.4%) tended to be more frequent in the older generation, even though the differences did not reach a statistical significance. No statistically significant difference was observed for the other phenotypic features. About 15% of the clinical features were diagnosed during the study.

Table 1 Clinical characteristics of second generation subjects and parents carrying the 22q11.2 deletion

	Second generation	Parents	
Total number of subjects	**32**	**26**	
	N (%)	**N (%)**	**p**
Facial anomalies	29 (90.6)	24 (92.3)	1
Congenital heart defect	20 (62.5)	2 (7)	**<0.0001**
Ca-P abnormalities	12 (37.5)	1 (3.8)	**0.0033**
Palatal anomalies	18 (56.2)	13 (50)	0.79
ENT anomalies	4 (12.5)	2 (7)	0.68
Renal disorders	7 (21.8)	2 (7)	0.16
Ocular disorders	1 (3.1)	6 (23)	**0.037**
Neurological disorders	3 (9.3)	1 (3.8)	0.62
Dental anomalies	8 (25)	10 (38.4)	0.39
Skeletal anomalies	15 (46.8)	10 (38.4)	0.6
Gastrointestinal disorders	8 (25)	2 (7)	0.16
Psychiatric disorders	4 (12.5)	9 (34.6)	0.06
Language delay	24 (75)	12 (46.1)	**0.031**
Developmental delay	23 (71.8)	11 (42.3)	**0.032**
Learning difficulty	23 (71.8)	16 (61.5)	0.57
Autoimmunity	4 (12.5)	5 (19.2)	0.71
Infections	9 (28.1)	3 (11.5)	0.19

Only severe infections (sepsis, pneumoniae), requiring hospitalization, or history of recurrent infections were considered. Bold indicate P values considered statistically significant.

We next compared the anatomic type of CHD between the 2 groups. We found that in the 20% of the subjects of the second generation with CHD, each individual patient had more than 1 abnormality, whereas in the parent group none of them had a more severe defect. A cyanotic CHD was found only in the 35% of the group with the cardiac defect. In particular, among these patients, we found that 7/20 subjects of the second generation exhibited a tetralogy of Fallot (TOF) and only 1/20 truncus arteriosus (TA). Among those with non cyanotic CHD, a patent ductus arteriosus (PDA) was observed in 4/20, atrial septal defects (ASD) in 4/20, interrupted aortic arch (IAA) in 4/20, ventricular septal defects (VSD) in 3/20, pulmonary valve stenosis (PVS) in 1/20 and other in 2/20. In the parent group, the anomalies found were a PDA in one case and a double aortic arch (DAA) in the other one.

With regard to calcium-phosphorus metabolism abnormalities, 9 subjects in the second generation presented with neonatal hypocalcemia, and in 2 of them a hypoparathyroidism was later diagnosed. Overall, at any age, a total of 5/32 (15.6%) of them received a full diagnosis of hypoparathyroidism. Only 1 subject of the parent group was affected with asymptomatic hypoparathyroidism.

Although the prevalence of the palatal defects was similar in the 2 groups (56 and 50%, respectively), we next compared the type of the defect and made a comparison. Within the group of patients of the second generation, 1 subject had cleft palate and bifid uvula, 16 velopharyngeal insufficiency, 4 hypernasal speech and 4 high arched palate. In the parent group, 11 had velopharyngeal insufficiency, 3 hypernasal speech, 1 high arched palate. Only in two parents, a cleft palate was observed. Thus, no remarkable difference was found for these variables.

Psychiatric disorders were more frequent in the parent group, even though the difference did not reach statistical significance ($P = 0.06$). We found that in the parent group, anxiety was observed in 5/26, mood disorders in 3/26, attention-deficit/hyperactivity disorders in 3/26, behavioral anomalies in 3/26, schizophrenia in 2/26, psychotic disorders not otherwise specified (NOS) in 2/26 and phobia in 1/26. Within the second generation group, 2 of them had behavioral abnormalities represented by trend to social isolation and rejection, impairment in social and daily living skills and low self-esteem, 1 also showed adaptive abnormalities, and the other one an attention deficit disorder. Schizophrenia was observed in 1 subject of the second generation, who, however, was 31 years-old.

Ocular defects were the only anomaly frequently observed in the parental generation, consisting in refractive defects (3/6), strabismus (2/6), retinal vessel abnormalities

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(1/6), cataract (1/6) and xerophthalmia (1/6). The only child with ocular defect had retinal vessels abnormalities.

Intrafamilial clinical phenotypic comparison

With regard to the intrafamilial phenotypic variability, in 23 out of the 32 child/parent couples a higher number of features was found in the second generation, although in 6 couples the number of features was higher in the parents' generation and in the remaining 3 couples no difference was found (Figure 1). None of the couples with higher number of features in the parents' generation had a CHD or hypoparathyroidism.

We then performed an intrafamilial evaluation of the clinical severity of the phenotype with regard to the prominent clinical features, whose prevalence was statistically different between the 2 groups, namely CHD, anomalies of the calcium-phosphorus metabolism, developmental and/or speech delay (Table 2).

In particular, we observed that CHD was present in 18 couples only in the second generation and, in one case, only in the first generation. In the couple in which both members were affected, the parent exhibited a PDA, whereas his child was affected with IAA, thus confirming the lack of phenotypic correspondence. Calcium-phosphorus abnormalities were found in 11 subjects of the second generation, and the only parent with asymptomatic hypoparathyroidism had a child who presented with hypocalcaemia. In 15 couples speech delay was exclusively observed in the second generation whereas in 5 couples only in the first generation. In the remaining 9 couples both members were affected. In 16 couples, developmental delay was exclusively found in the second generation, in 4 cases only in the first generation and in 8 couples both subjects were affected.

To rule out the interference of factors not related to the 22q11.2 deletion, in 6 families, data were collected also from the non-deleted parent. Of note, none of them had a CHD, while 2 children in this subgroup were affected. One subject reported learning and behavioral problems, but not intellectual disabilities, while in another case a borderline IQ of 68 was determined.

Furthermore, in the families with more than one affected child, a milder phenotype was observed in parents than in children, even though the 5 subjects of the second generation, who were diagnosed first, had a higher number of the major clinical features compared to their 6 siblings (16 core features vs 11).

Discussion and conclusions

We have compared the clinical phenotype of a cohort of 32 subjects affected with inherited 22q11.2DS and their transmitting parents.

In this study, we found a higher number of clinical features and a more severe phenotype in the second generation, which exhibited a higher number of more severe conditions. CHD, abnormalities of the calcium-phosphorus metabolism, developmental and/or speech delay were more represented in children than in parents. It should be considered that, in the past decades, severe CHD was associated with a high neonatal and infant mortality. This evidence was thought to have an appreciable impact on reducing the reproductive fitness of 22q11.2DS patients with CHD. More recently, however, with the improvement of cardiac surgery strategies, it is suggested

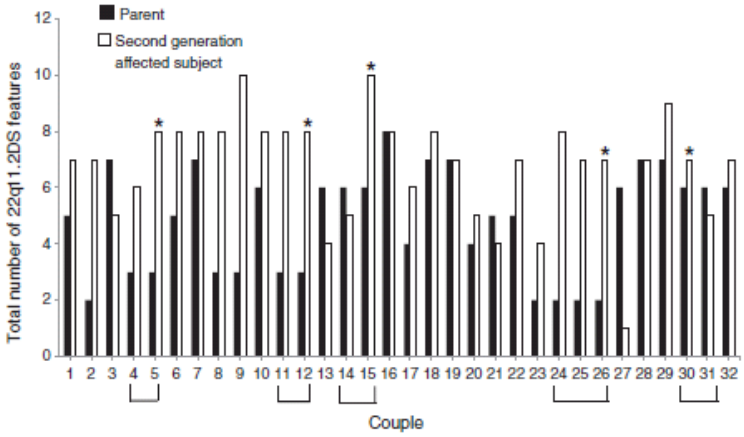

Figure 1 Intrafamilial phenotypic comparison. A higher number of features were observed in the second generation in 23 couples. In 6 couples the number of features was higher in the parents' generation and in the remaining 3 couples no difference was found. The brackets indicate the families with 2 or more second generation affected subjects. * indicates the proband within these families.

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Table 2 Intrafamilial comparison of the clinical severity of the phenotype

Couple	Congenital heart defect		Ca-P abnormalities		Speech delay		Developmental delay	
	Affected subject	Parent	Affected subject	Parent	Affected subject	Parent	Affected subject	Parent
1	yes	no	yes	yes	no	yes	yes	no
2	yes	no	yes	no	yes	no	no	no
3	yes	no	no	no	no	yes	yes	no
°4	no	no	yes	no	no	yes	yes	no
°5[δ]	yes	no	yes	no	yes	yes	yes	no
6	yes	no	no	no	yes	yes	yes	yes
7	yes	no	yes	no	yes	no	yes	no
8	no	no	no	no	yes	no	yes	no
9	no	no	no	no	yes	no	yes	no
10	no	yes	yes	yes	yes	yes	no	no
°11	yes	no	yes	no	no	no	yes	no
°12[δ]	yes	no	no	no	yes	no	yes	no
13	no	no	no	no	yes	no	no	yes
°14	no	no	no	no	yes	no	yes	yes
°15[δ]	yes	no	no	no	yes	no	no	yes
16	no	no	no	no	yes	no	yes	no
17	yes	no	no	no	yes	yes	yes	yes
18	yes	no	no	no	yes	yes	yes	yes
19	yes	no	no	no	yes	yes	no	yes
20	yes	yes	no	yes	yes	yes	yes	no
21	yes	no	yes	no	yes	yes	yes	yes
22	yes	no	yes	no	yes	yes	yes	yes
23	yes	no	yes	no	yes	no	yes	yes
°24	no	no	no	no	yes	no	yes	no
°25	no	no	no	no	yes	no	yes	no
°26[δ]	yes	no	yes	no	yes	no	yes	no
27	yes	no	yes	no	no	yes	no	yes
28	no	no	no	no	yes	yes	yes	yes
29	no	no	no	no	yes	no	yes	no
°30[δ]	yes	no	no	no	yes	no	yes	no
°31	no	no	no	no	no	no	no	no
32	yes	no	no	no	no	no	no	no

°Couple of siblings; [δ]First individual to seek medical attention for genetic evaluation.

that a stronger negative selective pressure against the transmission of 22q11.2 deletion is primarily due to the severity of the neuropsychiatric phenotype and intellectual disabilities [27].

The only anomalies more frequent in the parental generation were ocular abnomalities. Since most of them, in particular refractive defects and cataract usually develop during older age, we could not exclude a bias related to the different ages of the subjects in the two groups.

When an intrafamilial comparison of the phenotypic complexity was performed, a higher prevalence of clinical features were found in the second generation. When the comparison concerned the prominent clinical features, whose prevalence was statistically different between the 2 groups, namely CHD, calcium-phosphorus metabolism anomalies, developmental delay and speech delay, we observed that in almost all parent/child couples these major features were more frequent in the second generation. In previous studies [9,10,13-15,24], an intrafamilial

variability has already been reported, even though the comparison of intergenerational clinical features has not been performed. An ascertainment bias could partially explain this finding, since the first subject diagnosed within a family is likely to be more severely affected. Moreover, our observation may also be explained by a bias related to the low rate of reproductive fitness of adults with a more severe phenotype.

As to developmental delay, the presence of environmental intellectual disabilities may obviously *per se* influence the mental development of the offspring, because of psychosocial deprivation. However, it should be emphasized that in our cohort the majority of the subjects with developmental delay have a parent without intellectual disability. Moreover, in the 6 families in which also the non-deleted parent was studied, the lack of a clear correlation between environment and the child' development was noted. As expected, psychiatric disorders were more represented in the first generation, however, the difference was not significant thus suggesting the need of an accurate psychiatric management since childhood.

Several genes, such as *TBX1* [28], *HIRA*, *UFD1L* [29] and *CRKL* [30], within the 22q11 region have been considered to be implicated in the pathogenesis of the syndrome. The 22q11.2 phenotype is a developmental field defect, and a DiGeorge-like phenotype may also occur in the absence of the deletion [31], as in diabetic [32] and retinoic acid embryopathy, fetal alcohol syndrome, CHARGE [33] and Fraser syndromes, as well as other chromosomal anomalies, such as 10p13, 17p13, 4q34.1q35.2 [34-36], indicating that several molecules in a common genetic pathway or in functionally related pathways may be involved in 22q11.2DS clinical manifestations.

Several hypotheses have so far been proposed to explain intergenerational and intrafamilial phenotypic variability in genetic syndromes. Deletions of different sizes and location and the extension of an unstable mutation at the 22q11.2 locus could explain the clinical variability [37].

Evidence indicates that *TBX1* gene is sensitive to altered dosage [38], thus leading to the hypothesis that additional alterations of the other allele may explain the clinical variability. In humans, this does not seem to be the case, in that so far DNA variations in *TBX1* locus on the remaining allele were not found in 22q11.2DS patients exhibiting a variable cardiovascular expression and palatal defects. Thus, it is likely that gene modifiers not related to chromosome 22 may be implicated [39,40].

The increased risk of cardiac defects observed in unaffected relatives of 22q11.2DS subjects with CHD, suggested a potential role for genes outside the DiGeorge critical region [41,42]. However, studies aimed at identifying genetic factors outside of the 22q11,2 region, such as *VEGFA* [43] or folate-related genes [44] failed to reveal any association. It should be noted that in the subgroup of families in whom also the non-deleted parent was studied, no CHD was noted suggesting the absence of interfering genetic factors not related to the 22q11.2 deletion in this context.

A copy-number variation may explain a reduced penetrance of some disease-causing mutations [45]. A genetic compensatory effect has also been documented in families of 22q11.2DS subjects, whose clinically normal parent carried 22q11.2 deletion compensated by an insertion of the 22q11.2 critical region inside the other copy of the chromosome [46]. Finally, a mosaic status in the carrier parent, even though rare, could be the explanation of the variable and more benign phenotype. At the moment, we cannot yet exclude the presence of a concomitant duplication or copy number variations in the former generation, since further studies with interphase FISH or array-CGH are required to rule out this hypothesis.

During development, gene expression is accurately orchestrated in time and space in a program that involves enzymes controlling nucleosome remodeling, histone modification and DNA methylation [47]. A demonstration that an epigenetic alteration could result in a DiGeorge syndrome phenocopy has been recently documented in mutant mice lacking the MOZ histone acetyltransferase [48]. Thus, failure of these fine tuned mechanisms could result in an interference of the phenotypic expression.

In our study we also observed that some clinical features were more represented in the previous generation, although the difference did not reach the statistical significance for the limited number of subjects studied. In keeping with recent findings, we observed a higher incidence of psychiatric disorders in the older generation.

The identification of adults with a milder phenotype deserves careful attention since a later onset illness associated with 22q11.2DS has been reported, highlighting the possibility that along with psychiatric disorders, also treatable conditions such as symptomatic or asymptomatic hypocalcaemia, thrombocytopenia and hypothyroidism may occur [49]. Early recognition of these features [50] could provide the benefit of an early treatment [51-53].

Advantages and limitations of the study

This is the largest cohort of subjects affected with familial 22q11.2DS. A detailed characterization of the clinical features of such subjects, and an intergenerational/intrafamilial clinical comparison has been performed. The observation that within the families, the patients who were first diagnosed had a higher number of core features as compared to their siblings or parents would suggest an ascertainment bias, even though the clinical phenotype of the parents was milder compared to their children. Another possible explanation could be the co-inheritance of a further genetic defect from the non-affected parent. This seems unlikely since this co-inheritance should have

occurred in all cases exhibiting the worsening of the phenotype. As for the CHD, it should be noted that none of the non-affected parents had a CHD, thus excluding, at least for this feature, this hypothesis.

Additional file

Additional file 1: Table S1. Demographic characteristics of the 22q11.2DS subjects.

Abbreviations

22q11DS: 22q11.2 Deletion syndrome; LCR: Low-copy repeats; IPINET: Italian network for primary immunodeficiencies; WISC: Wechsler intelligence scale for children; WAIS: Wechsler adult Intelligence Scale; FISH: Fluorescence in situ hybridization; MLPA: Multiplex ligation-dependent probe amplification; CGH: Comparative genoma hybridization; CHD: Congenital heart defect; TOF: Tetralogy of Fallot; TA: Truncus Arteriosus; PDA: Patent Ductus Arteriosus; ASD: Atrial septal defects; IAA: Interrupted Aortic Arch; VSD: Ventricular septal defects; PVS: Pulmonary valve stenosis; DAA: Double aortic arch.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CE conceptualized and designed the study, enrolled patients and collected data, drafted the initial manuscript. GG, VG enrolled patients, collected data and coordinated data collection from each group, drafted the initial manuscript. PP, CA, FB, MPC, RC, SM, BM, CM, VM, AP, GS,CC, PR enrolled patients, collected the data, and revised the manuscript. MCD enrolled patients, collected data and critically revised the manuscript. CP conceptualized and designed the study, enrolled patients, collected data and drafted the initial manuscript. MCD and CP equally contributed to the paper. All the authors approved the final version of the manuscript.

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Author details

[1]Department of Translational Medicine, "Federico II" University, Naples, Italy. [2]Department of Pediatrics, (DPUO), University of Rome Tor Vergata, Rome, Italy. [3]Department of Pediatrics, Anna Meyer Children's University Hospital, Florence, Italy. [4]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Milan; Pediatric ImmunoHematology IRCCS San Raffaele Hospital, Milan, Italy. [5]Department of Pediatrics, Giovanni XXIII Pediatric Hospital, Bari, Italy. [6]Pediatric ImmunoHematology IRCCS, San Raffaele Hospital, Milan, Italy. [7]Department of Internal and Experimental Medicine, University of Pisa, Pisa, Italy. [8]Department of Pediatrics, University of Turin, Turin, Italy. [9]Department of Biomedicine and Evolutive Aging, University of Bari, Bari, Italy. [10]A. Nocivelli Institute for Molecular Medicine, Pediatric Clinic, University of Brescia, Brescia, Italy. [11]Medical Genetics Unit, General Hospital G. Rummo, Benevento, Italy. [12]Medical Genetics, Bambino Gesù Pediatric Hospital, IRCCS, Rome, Italy. [13]Department of Translational Medical Sciences, Unit of Pediatric Immunology, "Federico II" University, via S. Pansini, 5-80131 Naples, Italy.

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Chapter 6

“Other genetic diseases treated by lentiviral gene therapy approaches”

RESEARCH ARTICLE SUMMARY

Lentiviral Hematopoietic Stem Cell Gene Therapy Benefits Metachromatic Leukodystrophy

Alessandra Biffi,* Eugenio Montini, Laura Lorioli, Martina Cesani, Francesca Fumagalli, Tiziana Plati, Cristina Baldoli, Sabata Martino, Andrea Calabria, Sabrina Canale, Fabrizio Benedicenti, Giuliana Vallanti, Luca Biasco, Simone Leo, Nabil Kabbara, Gianluigi Zanetti, William B. Rizzo, Nalini A. L. Mehta, Maria Pia Cicalese, Miriam Casiraghi, Jaap J. Boelens, Ubaldo Del Carro, David J. Dow, Manfred Schmidt, Andrea Assanelli, Victor Neduva, Clelia Di Serio, Elia Stupka, Jason Gardner, Christof von Kalle, Claudio Bordignon, Fabio Ciceri, Attilio Rovelli, Maria Grazia Roncarolo, Alessandro Aiuti, Maria Sessa, Luigi Naldini*

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http://dx.doi.org/10.1126/science.1233158

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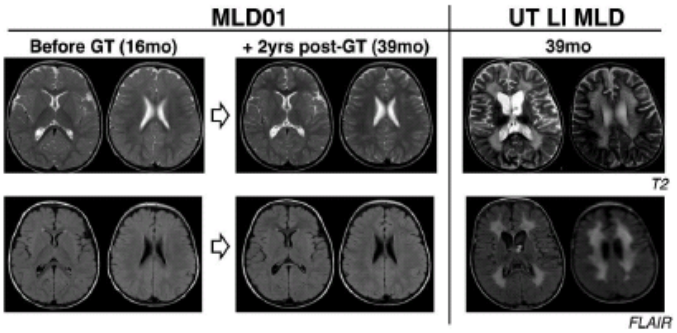
Introduction: Metachromatic leukodystrophy (MLD) is a neurodegenerative lysosomal storage disease caused by arylsulfatase A (ARSA) deficiency. The disease primarily affects children and invariably leads to premature death. In previous work with a mouse model of MLD, we used a lentiviral vector (LV) to introduce a functional *ARSA* gene into hematopoietic stem cells (HSCs) ex vivo and showed that reinfusion of the engineered HSCs prevented and corrected disease manifestations in the animals. To determine whether this gene therapy strategy is safe and can offer therapeutic benefit to patients with early-onset MLD, we designed a phase I/II trial.

Methods: We optimized LV manufacturing and HSC transduction in clinical grade conditions. Three children with ARSA deficiency and mutations associated with early-onset MLD were treated at the presymptomatic stage; all had at least one older sibling affected by the same disease variant. HSCs from the patients were transduced ex vivo with a LV carrying the *ARSA* gene.

Patients were treated with a myeloablative busulfan conditioning regimen before reinfusion of the engineered HSCs. Clinical and objective evaluations were collected up to 24 months after treatment. Molecular follow-up of vector integration site distribution was performed on hematopoietic cells derived from bone marrow and peripheral blood.

Results: There was high-level stable engraftment of the transduced HSCs in the bone marrow and peripheral blood of all patients at all times tested, with 45 to 80% of bone marrow–derived hematopoietic colonies harboring the vector. With these high gene marking levels, ARSA activity was reconstituted to above normal values in the hematopoietic lineages and in the cerebrospinal fluid. Analysis of >36,000 different LV integration sites showed that the high gene marking was sustained by highly polyclonal engraftment of transduced cells without evidence of aberrant clonal behavior. Several integration sites were shared among progenitors and mature myeloid, B- and T-cells sampled at long intervals after treatment, indicating efficient transduction and engraftment of HSCs. These findings were associated with a clear therapeutic benefit because the disease did not progress in any of the treated patients, even after the time of onset projected from sibling cases.

Discussion: Our gene therapy protocol allows stable engraftment of transduced HSCs at high levels and without evidence of vector-induced genotoxicity. The reconstitution of ARSA activity in the cerebrospinal fluid and the arrested progression of neurodegenerative disease in the three treated patients demonstrate that the transplanted cells, or their progeny, can seed the nervous system and deliver therapeutic levels of active enzyme. Although our data are promising, long-term follow-up of the patients is needed in order to establish the full therapeutic potential of this gene therapy strategy for MLD. In addition, our data position LV gene transfer as a feasible means to engineer human hematopoiesis to its near entirety—an approach that could be exploited for treatment of other diseases.



HSC gene therapy can prevent progression of metachromatic leukodystrophy. Magnetic resonance (MR) images of the brain of a patient (MLD01) before and after gene therapy. The brain of this patient appeared largely normal 2 years after treatment. In contrast, the brain of an untreated, age-matched late infantile MLD patient (UT LI MLD) showed severe demyelination associated with diffuse atrophy. (**Top**) Axial T2 weighted fast spin-echo MR images. (**Bottom**) Fluid-attenuated inversion recovery (FLAIR) MR images.

FIGURES IN THE FULL ARTICLE

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.1233158/DC1
Materials and Methods
Figs. S1 to S22
Tables S1 to S19
References

RELATED ITEMS IN *SCIENCE*

Aiuti *et al.*, Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* **341**, 1233151 (2013). doi:10.1126/science.1233151

I. Verma, Edging toward the promise of gene therapy. *Science* **341**, 853–855 (2013). doi:10.1126/science.1242551

The list of author affiliations is available in the full article online.
*Corresponding author. E-mail: biffi.alessandra@hsr.it (A.B.); naldini.luigi@hsr.it (L.N.)

RESEARCH ARTICLE

Lentiviral Hematopoietic Stem Cell Gene Therapy Benefits Metachromatic Leukodystrophy

Alessandra Biffi,[1,2,3]*† Eugenio Montini,[1]* Laura Lorioli,[1,2,3,4] Martina Cesani,[1] Francesca Fumagalli,[2,4,5] Tiziana Plati,[1] Cristina Baldoli,[6] Sabata Martino,[7] Andrea Calabria,[1] Sabrina Canale,[2] Fabrizio Benedicenti,[1] Giuliana Vallanti,[8] Luca Biasco,[1] Simone Leo,[9] Nabil Kabbara,[10] Gianluigi Zanetti,[9] William B. Rizzo,[11] Nalini A. L. Mehta,[12] Maria Pia Cicalese,[2,3] Miriam Casiraghi,[2] Jaap J. Boelens,[13] Ubaldo Del Carro,[5] David J. Dow,[12] Manfred Schmidt,[14] Andrea Assanelli,[3,15] Victor Neduva,[12] Clelia Di Serio,[4] Elia Stupka,[16] Jason Gardner,[17] Christof von Kalle,[14] Claudio Bordignon,[4,8] Fabio Ciceri,[3,15] Attilio Rovelli,[18] Maria Grazia Roncarolo,[1,2,3,4] Alessandro Aiuti,[1,2,3,19] Maria Sessa,[2,5] Luigi Naldini[1,4]†

Metachromatic leukodystrophy (MLD) is an inherited lysosomal storage disease caused by arylsulfatase A (ARSA) deficiency. Patients with MLD exhibit progressive motor and cognitive impairment and die within a few years of symptom onset. We used a lentiviral vector to transfer a functional *ARSA* gene into hematopoietic stem cells (HSCs) from three presymptomatic patients who showed genetic, biochemical, and neurophysiological evidence of late infantile MLD. After reinfusion of the gene-corrected HSCs, the patients showed extensive and stable *ARSA* gene replacement, which led to high enzyme expression throughout hematopoietic lineages and in cerebrospinal fluid. Analyses of vector integrations revealed no evidence of aberrant clonal behavior. The disease did not manifest or progress in the three patients 7 to 21 months beyond the predicted age of symptom onset. These findings indicate that extensive genetic engineering of human hematopoiesis can be achieved with lentiviral vectors and that this approach may offer therapeutic benefit for MLD patients.

Metachromatic leukodystrophy (MLD) is an autosomal recessive lysosomal storage disease caused by mutations in the *ARSA* gene that result in a deficiency of the enzyme arylsulfatase A (*1*). ARSA deficiency causes accumulation of the enzyme substrate sulfatide in oligodendrocytes, microglia, and certain neurons of the central nervous system (CNS), and in Schwann cells and macrophages of the peripheral nervous system (PNS). This build-up of sulfatide leads to widespread demyelination and neurodegeneration, which is ultimately manifested in patients as severe progressive motor and cognitive impairment. MLD is classified into clinical variants according to the age of symptom onset. Patients with the late-infantile (LI) variant show symptoms within the second year of life, have the most severe manifestations of the disease, and die within a few years of symptom onset. Current treatments do not substantially delay the progression or fatal outcome of MLD (*2*, *3*).

Hematopoietic stem cell transplantation (HSCT) and HSC gene therapy (HSC-GT) have been investigated for the treatment of lysosomal storage diseases because enzyme-proficient hematopoietic progeny cells can migrate to the affected tissues, including the CNS, scavenge the stored material, such as sulfatide in MLD, and cross-correct the resident cells (*4*). The efficacy of these treatments varies among different types of lysosomal storage diseases (*3*, *5*–*7*) and with the extent of functional gene expression in the transplanted cells (*8*–*11*). Achieving a high level of functional gene replacement is particularly important in the setting of HSC-GT; to date, gene replacement in the hematopoietic cells of treated patients has been limited (*12*–*15*).

In a mouse model of MLD, we have demonstrated that disease manifestations can be prevented and corrected with lentiviral vector (LV)–based HSC-GT but not HSCT (*8*, *9*, *16*). This is consistent with the observation that HSCT fails to provide consistent benefits in MLD patients (*3*, *5*–*7*). LV-based HSC-GT induced extensive and supraphysiological expression of the functional *ARSA* gene throughout the HSC progeny, which in turn mediated widespread cross-correction of CNS and PNS resident cells (*8*, *9*).

Translating this strategy into a clinical protocol, however, posed several challenges, including gene transfer efficacy and safety. Although stable marking (10 to 15%) was reported in two patients with X-linked adrenoleukodystrophy (X-ALD) in the first trial of LV-mediated HSC-GT (*17*), it was not clear whether LVs could transduce human HSCs in clinically relevant settings with the efficiency required for application to MLD gene therapy. Vector safety is also a concern in light of the clinical experience with γ-retroviral vectors, which showed that genome-wide vector integration in hematopoietic stem/progenitor cells (HSPCs) has the potential to trigger leukemia through insertional mutagenesis (*14*, *18*). Although several studies have shown that LVs reduce the risk of genotoxic insertions because of their advanced design and the integration site selection typical of the parental HIV (*19*, *20*), it remained possible that increasing the total integration load in the infused HSPCs would offset the biosafety advantage.

In the present study, we optimized LV-mediated gene transfer into human HSCs for clinical translation and used this HSC-GT protocol to treat nine patients with early onset MLD in a phase I/II trial. Here, we report the outcome of the treatment in the first three treated patients at 24 months follow-up for patient MLD01 and 18 months follow-up for patients MLD02 and MLD03.

Results

Efficient ex Vivo Transfer of the ARSA Gene into the HSPCs of MLD Patients

Transduction of human bone marrow (BM)–derived $CD34^+$ cells was optimized to reach ≥2 vector copy number per genome (VCN) (fig. S1), based on the ARSA overexpression levels required for therapeutic efficacy in preclinical studies (*8*, *9*, *21*, *22*). $CD34^+$ cells were stimulated ex vivo with early acting cytokines in serum-free medium and transduced with purified third-generation LVs encoding the human ARSA cDNA under the control of the human phosphoglycerate kinase promoter (PGK) (fig. S2). VCN was measured in the progeny of the treated cells obtained by means of bulk liquid culture, colony-forming assays and repopulation of chimeric $Rag2^{-/-}$ $\gamma chain^{-/-}$ mice.

[1]San Raffaele Telethon Institute for Gene Therapy (TIGET), San Raffaele Scientific Institute, 20132 Milan, Italy. [2]TIGET Pediatric Clinical Research Unit, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, 20132 Milan, Italy. [3]Pediatric Immunohematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, 20132 Milan, Italy. [4]Vita-Salute San Raffaele University, 20132 Milan, Italy. [5]Neurology Unit, Department of Neurology, San Raffaele Scientific Institute, 20132 Milan, Italy. [6]Neuroradiology Unit, Head and Neck Department, San Raffaele Scientific Institute, 20132 Milan, Italy. [7]Department of Experimental Medicine and Biochemical Sciences, University of Perugia, 06122 Perugia, Italy. [8]MolMed, 20132 Milan, Italy. [9]Distributed Computing Group, Center for Advanced Studies, Research and Development in Sardinia (CRS4), 09010 Pula, Italy. [10]Pediatric Hematology Oncology Division, Rafic Hariri University Hospital, Beirut, Lebanon. [11]Department of Pediatrics, University of Nebraska Medical Center, Omaha, NE 68198, USA. [12]Molecular and Cellular Technologies, GlaxoSmithKline, Stevenage SG1 2NY, UK. [13]Pediatric Blood and Marrow Transplantation Program, University Medical Center 3584 CX Utrecht, Netherlands. [14]Department of Translational Oncology, National Center for Tumor Diseases and German Cancer Research Center, 69120 Heidelberg, Germany. [15]Hematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, 20132 Milan, Italy. [16]Center for Translational Genomics and Bioinformatics, San Raffaele Scientific Institute, 20132 Milan, Italy. [17]Regenerative Medicine Discovery Performance Unit, GlaxoSmithKline Research and Development, King of Prussia, PA 19406, USA. [18]Bone Marrow Transplant Unit, MBBM Foundation, Pediatric Department, Milano-Bicocca University at San Gerardo Hospital, 20052 Monza, Italy. [19]University of Rome Tor Vergata, 00133 Rome, Italy.

*These authors contributed equally to this work.
†Corresponding author. E-mail: biffi.alessandra@hsr.it (A.B.); naldini.luigi@hsr.it (L.N.)

RESEARCH ARTICLE

A summary of the LV manufacturing process (flow chart, main steps, and yields) as well as the specifications set for testing the good manufacturing practice (GMP) lots released for this study are reported in fig. S3 and tables S1 and S2.

After optimizing the gene transfer procedures for human cells, we designed a clinical trial of HSC-GT in which autologous HSPCs from patients with MLD would be transduced ex vivo with ARSA-encoding LVs and then reinfused after the patients had been treated by a myeloablative regimen using the alkylating agent busulfan (fig. S4). Because MLD patients usually express residual, nonfunctional ARSA protein, they are unlikely to mount an immune response toward ARSA. Our trial design therefore did not include immunosuppressive agents.

Three presymptomatic MLD patients, biochemically characterized for ARSA deficiency and carrying mutations associated with LI MLD, were enrolled and treated; all patients had one or more older siblings with LI MLD onset within 2 years of age (table S3). At enrollment, patients were defined as presymptomatic based on the lack of obvious clinical signs of the disease, independent of the presence of abnormalities in instrumental tests [electroneurographic recordings (ENG) and/or brain magnetic resonance imaging (MRI)]. Treatment of the patients began 2 to 12 months before the reported age of onset of the disease in their affected matched siblings (*23*–*25*).

Twenty to 30 days after collection of an HSC back-up, a BM harvest was performed followed by isolation of $CD34^+$ cells by means of standard immunomagnetic procedures. Transduction of patients' $CD34^+$ cells was performed as described above. Cells were washed, resuspended, and kept at +4°C until successful completion of a first panel of fast quality control (QC) tests (table S4). The transduced $CD34^+$ cells were then released and infused fresh intravenously. Other relevant QC results (table S4) became available 2 to 5 weeks after infusion. VCN ranged from 2.5 to 4.4, transduction efficiency was 90 to 97%, and ARSA activity was reconstituted at ≥10-fold the level measured in healthy controls in the cultured progeny of the infused cells (fig. S1 and table S5).

A myeloablative, dose-adjusted busulfan regimen [target area under the curve (AUC) 4800 μg/L*hour] (table S5) was administered intravenously to the patients from day −4 up to day −1 before HSC-GT in a total of 14 doses, with the last busulfan dose administered ≥24 hours before transduced cell infusion. The conditioning regimen was well tolerated. The patients experienced severe neutropenia (absolute neutrophil count < 500/μl) from as early as day +9 up to day +45 after transplant at the latest (fig. S5 and table S5). No reduction or only a minor transient reduction of lymphocyte counts was observed in the absence of immunosuppressive/lymphotoxic drugs in the pretransplant conditioning (fig. S5). Patients required transfusional support up to day +45 after treatment for thrombocytopenia and anemia (fig. S5). Thereafter, the patients rapidly recovered hematological parameters matching the normal values for age. No abnormal expansion or clonal outgrowth was detected in the peripheral blood (PB) and BM by cell type composition, cytologic, karyotypic, or immunophenotypic studies (fig. S5). A mild (maximum National Cancer Institute/National Institutes of Health common toxicity criteria grade 2) transient increase in hepatocellular enzyme levels was detected within 2 to 3 weeks after treatment. Serious adverse events included two cases of central venous catheter–related infection, which promptly resolved upon antibiotic treatment. Replication competent LVs (RCLs), antibodies to HIV Gag p24, and antibodies to ARSA in patients' PB were negative at all times tested.

High-Level Engraftment of the Transduced HSPCs and Sustained ARSA Reconstitution

Beginning 1 month after transplant, we observed high-level stable engraftment of the transduced cells in the BM and PB of all patients at all times tested (Fig. 1A). Between 45 and 80% of the colonies outgrown from patients' BM in the colony-forming cell (CFC) assay harbored the LV genome (Fig. 1A). Quantitative polymerase chain reaction (PCR) on $CD34^+$ cells sorted from the patients' BM showed stable gene marking with VCN ranging from 0.9 to 1.9 up to the latest follow-up time (Fig. 1B). Considering the fraction of cells harboring the vector (estimated from the percentage of clonogenic progenitors outgrown from the patients' BM positive for LV DNA), these values imply a VCN of 2 to 4 in the engrafted transduced $CD34^+$ cells, which closely matches the data measured on the in vitro progeny of the infused cells. Correspondingly high and stable gene marking was observed in the circulating $CD15^+$ granulocytes and $CD14^+$ monocytes (Fig. 1C), and in myeloid cells and progenitors isolated from the BM (fig. S6), beginning with the first sampling and throughout the follow-up. A progressive increase in gene marking of lymphoid cells was observed—more rapid for B and NK cells and delayed for the longer-lived T cells—as expected from the choice of the conditioning regimen (Fig. 1C and fig. S6). The different repopulation kinetics of hematopoietic lineages by the transduced HSCs accounts for the progressive increase of the gene-marking level observed in total PB mononuclear cells (PBMCs) (Fig. 1D).

The high level of gene marking drove reconstitution of the defective ARSA activity up to above-normal values in the most therapeutically relevant myeloid populations (Fig. 2, A and B) as well as in other circulating cells (fig. S7, A and B). ARSA protein was isolated from patient hematopoietic cells as early as 1 month after treatment (fig. S7C) at above-normal expression levels in all tested samples (fig. S7D). The enzyme isolated from patient cells was found to hydrolyze the natural substrate sulfatide in vitro, confirming full functionality (0.003 mU of ARSA isolated from normal donor and treated patients' hematopoietic cells hydrolyzed 3.6 ± 1.2 nmol of substrate). In situ hybridization for LV mRNA and immunofluorescence for ARSA expression costained a high proportion of patients' blood cells consistently with the high VCN detected by means of quantitative PCR in these cells (fig. S8 and table S6). Func-

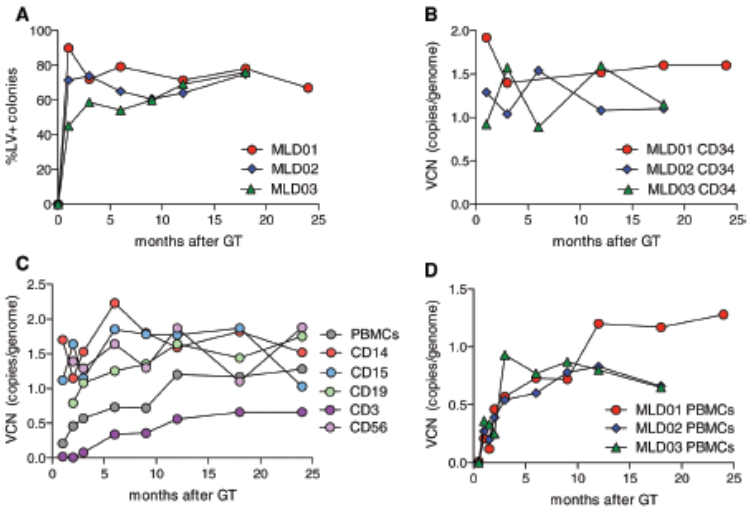


Fig. 1. Gene marking in patients after HSC-GT. (**A**) Engraftment of the transduced cells, evaluated with quantitative PCR on individual colonies from CFC assay performed on PB- and BM-derived cells and expressed as percentage (%) of LV^+ colonies on total tested colonies. (**B** to **D**) VCN expressed as copies of LV/human genome measured with quantitative PCR on BM-derived $CD34^+$ cells (B), individual subpopulations isolated from PB of patient MLD01 (C), and total PBMCs from patients MLD01, -02 and -03 (D).

tional ARSA was isolated from cerebrospinal fluid (CSF) collected from all three patients 1 year (and 2 years for MLD01) after HSC-GT at levels and activity comparable with those obtained from healthy donor samples, whereas no ARSA protein could be isolated from CSF before HSC-GT treatment (Fig. 2, C and D). These data demonstrate stable above-normal ARSA expression throughout the hematopoietic lineages and efficient delivery and bioavailability of the enzyme in the CNS after HSC-GT.

HSC-GT Provides Therapeutic Benefit to MLD Patients

Clinical observation and objective evaluations were collected up to 24 months after treatment for MLD01 and up to 18 months after treatment for MLD02 and MLD03. At the last follow-up, MLD01, MLD02, and MLD03 were 39, 30, and 25 months old, respectively (table S3). Because disease onset in the LI variant occurs invariably before 24 months of age and clinical manifestations are highly homogeneous, in particular among siblings (*23*, *24*), all three patients in our study were evaluated beyond their expected age of disease onset.

MLD01 is particularly informative because the follow-up time for this patient is longest and because the predicted time of symptom onset was earlier than that of the other two patients. This patient's two older siblings manifested regression of psychomotor performance and arrest of acquisition of new motor and cognitive skills at 18 months of age. Consistent with LI-MLD natural history (*26*), both siblings had rapid disease progression after onset and reached level 6 of the Gross Motor Function Scale for MLD (GMF-C MLD) (*27*) by 30 months of age, when they were wheelchair-bound and unable to support their head and trunk (table S7). In contrast, at 39 months of age MLD01 was able to stand independently and to walk and run with single aid (either the hand of another individual or an external walking device) (level 2 of the GMF-C MLD scale) (table S7). His Gross Motor Function Measure (GMFM) score progressively increased from enrollment (GMFM score of 164) up to a score of 193 at 39 months, which was substantially higher than all the scores recorded in our age-matched historical LI-MLD cohort [5.5 on average (*26*)], suggesting a continuous motor development (Fig. 3A and table S8). Cognitive evaluation by the Bayley Scale of Infant and Toddler Development revealed a normal composite IQ score for chronological age, with normal language and cognitive abilities (table S9) at all tested time points, including the last one at 39 months of age, an age at which the two affected siblings were incapable of any voluntary speech. The preexisting severe peripheral neuropathy of MLD01 [nerve conduction velocity (NCV) index (*26*) –11.5 at baseline] improved after treatment (NCV index –7.1 at last follow-up) (Fig. 3B). Brain MRI showed slight signal inhomogeneity, present since baseline evaluation and stable thereafter, and a small area of hyper-intensity in the corpus callosum that appeared at the 12-month follow-up and remained stable thereafter. All other brain MR findings were normal for age (Fig. 3C). In contrast, untreated LI-MLD patients invariably develop extensive and severe demyelination associated with diffuse atrophy by 24 to 36 months of age (Fig. 3C) (*26*, *28*). To our knowledge, no unequivocally diagnosed LI-MLD patient at the age of 39 months has been reported to display clinical features as positive as those we observed in MLD01.

Patients MLD02 and MLD03 remain fully asymptomatic, with normal motor and cognitive development for their ages (30 and 25 months, respectively); however, the clinical follow-up period for these patients after the predicted time of symptom onset was shorter than that for MLD01. At the latest clinical follow-up (18 months after treatment), MLD02 was 7 months beyond and MLD03 was 10 months beyond the predicted time of symptom onset. Both patients had a GMF-C MLD score of 0 at the last evaluation, which is in sharp contrast with the disease evolution documented in their affected siblings at the corresponding age (table S7). Their GMFM scores progressively increased after treatment, which is consistent with the acquisition of normal developmental motor milestones for age (Fig. 3A and table S8). Furthermore, in both patients the Bayley Scale of Infant and Toddler Development showed a normal composite IQ score for the age at all times tested (table S9). The ENG findings for both patients remained stable from baseline up to the latest follow-up; MLD02 showed near normal motor and sensory conduction velocities since baseline, whereas MLD03 showed a stable mild demyelinating neuropathy (Fig. 3B). Brain MRI revealed that both patients had a normal progression of white matter signal due to myelination up to the latest follow-up. MLD02 had small areas of T2 and fluid-attenuated inversion recovery (FLAIR) hyper-intensity signal in posterior and anterior periventricular white matter, present since baseline and stable up to the latest follow-up (fig. S9).

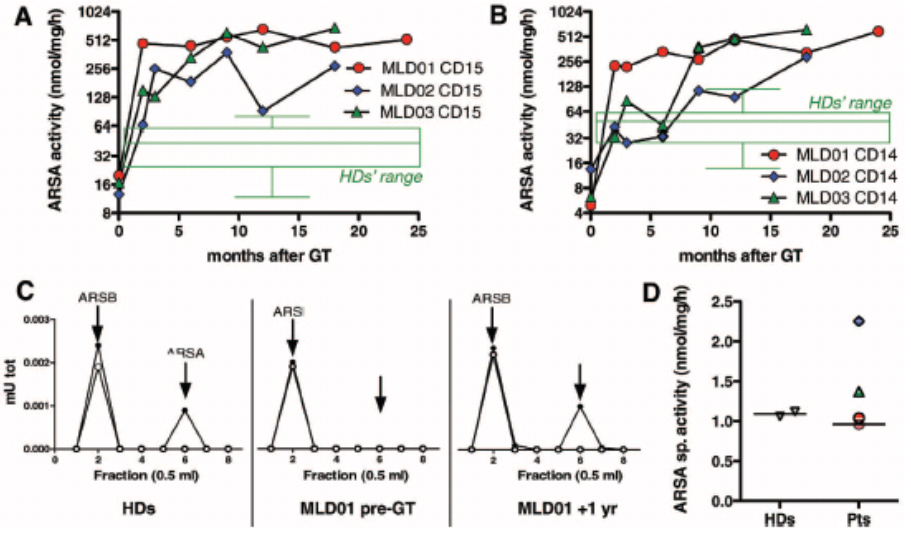


Fig. 2. ARSA expression in patients after HSC-GT. (**A** and **B**) ARSA activity measured with the *p*-nitrocatechol sulfate (PNC) assay on $CD15^+$ cells (A) and $CD14^+$ cells (B) isolated from the patients' PB. The activity range measured in a cohort of healthy donors (HDs) ($n \geq 10$ subjects) is shown. (**C**) Representative DEAE cellulose-chromatography analysis on 500 μl of cerebrospinal fluid (CSF) from a pool of four HDs, of a representative MLD patient before treatment (MLD01 pre-GT) and of the same patient 1 year after gene therapy. The peak of activity corresponds to the native form of the ARSA enzyme, as also demonstrated by its absence/very low residual activity in the patient's pretreatment sample. (**D**) Specific activity (toward MUS) of the ARSA enzyme isolated from the CSF of HDs (two cohorts of four donors each) and of the treated MLD patients (circles, MLD01; blue diamond, MLD 02; and green triangle, MLD03) sampled 12 months (red circle, MLD01; MLD02 and -03 were also sampled at 12 months) and 24 months (pink circle, MLD01) after treatment.

RESEARCH ARTICLE

Polyclonal Reconstitution of Hematopoiesis Without Evidence of Vector Genotoxicity

To determine whether there was any cause for concern regarding the safety of the LV in these patients, we monitored the clonal dynamics of hematopoietic repopulation at the molecular level by analyzing the vector genomic integrations in the patients' reconstituted hematopoietic cells (figs. S10 and S11, table S10, and materials and methods). Because of the genome-wide quasi–random integration profile of LV, each insertion creates a different genetic marker that can be used to track the clonal behavior of individual transduced cells. Genomic DNA was obtained from whole BM, PB cells, and subsets (table S11) at 1, 3, 6, 9, and 12 months for all patients and, for MLD01 and MLD02, 18 months after HSC-GT (table S12). Vector-genome junctions were retrieved by means of linear amplification mediated–PCR (LAM-PCR) by using three different restriction enzymes (fig. S12). From the three patients, >800 independent LAM-PCRs were generated and sequenced by 454-Roche (Basel, Switzerland) and Illumina (San Diego, California) technologies to obtain >10×10^6 sequence reads. Using a bioinformatics analysis pipeline and collision/contamination filtering (fig. S10, fig. S13, and table S13, A to C), we mapped integration sites (IS) for patient MLD01 (1,120,414), MLD02 (1,518,802), and MLD03 (425,356) on the human genome (NCBI HG19). Because many identical ISs were retrieved from multiple lineages and/or time points, the number of distinct ISs was 14,482, 11,077, and 10,959 for patients MLD01, MLD02 and MLD03, respectively. The genomic distribution of IS, both in the in vitro–cultured $CD34^+$ cells and in vivo, matched the previously reported LV preference for integration within transcriptional units (on average, 80% of IS were within genes) (fig. S14). To identify the gene classes preferentially targeted by LV integrations, we analyzed IS data sets from either the in vitro progeny of the infused $CD34^+$ cells or a pool of all in vivo–harvested samples for each patient using the GREAT (Genomic Regions Enrichment of Annotations Tool) software (table S14, A to F). In all patients, the gene classes of chromatin modification/remodeling, major histocompatibility complex class II–related functions, steroid hormone receptors, and RNA processing were overrepresented (table S14, A to F). These gene classes were the same, or shared >75% genes, with gene classes overrepresented in the ALD HSC-GT (*17*, *29*) (Fig. 4A and fig. S15).

We measured the proportion of sequencing reads representing each IS within our data sets as a surrogate readout for the relative abundance of the cell clone harboring that integration at a given time. For each time point, we analyzed the IS data sets from the $CD34^+$, myeloid (pooled $CD13^+$, $CD14^+$, and $CD15^+$ data sets), B ($CD19^+$) and T cells ($CD3^+$). Almost every cell clone marked by a specific IS accounted for only a fraction of a percent of the total clones at any given time. A few IS-marked cell clones showed a higher percentage at one time point but then disappeared or were strongly reduced at later time points (Fig. 4, B and C, and fig. S16). From these results, we conclude that no clonal dominance events occurred in these patients.

To determine whether other hallmarks of insertional mutagenesis were present in the patients, we assessed the occurrence of common insertion sites (CISs), insertional hotspots that may result from integration bias at the time of transduction or in vivo selection of clones harboring integrations that confer growth advantage. CISs were identified with an algorithm based on Abel *et al.* (*30*) and by the Grubbs test for outliers (*29*). Both analyses showed that the MLD CISs were mostly distributed in clusters within gene-dense regions heavily targeted by LV integrations (Fig. 5A, figs. S17 to S19, and tables S15 and S16), many of which had already been described in the ALD HSC-GT trial (Fig. 5B) (*17*, *29*). Given the larger size of the MLD data set, it contained almost the entire ALD CIS data set. Moreover, the Grubbs test for outliers when applied to the genomic regions surrounding each CIS confirmed that the CIS from the MLD clinical trial were not significantly over-targeted with respect to the neighboring genes, with *OPTC* being the only exception (fig. S19F and table S16). Cell clones with IS targeting CIS genes did not represent the most

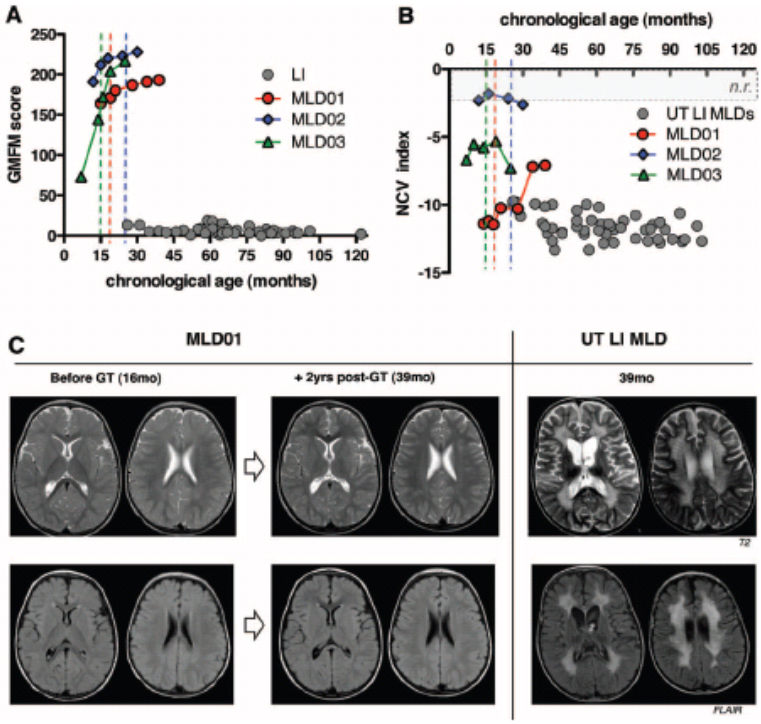


Fig. 3. Clinical follow up of MLD patients after HSC-GT. (**A** and **B**) GMFM score (A) and NCV index (B) of the three treated patients and of a historical cohort of LI-MLD patients (gray circles). The dotted lines indicate for each treated patient (inset, color code) the expected time of disease onset, according to the disease onset observed in their affected matched siblings; n.r., normal range of the NCV index. (**C**) Axial T2 weighted fast spin-echo MR images (top) and FLAIR MR images (bottom) obtained from patient MLD01 at baseline (before GT) and at +2 years after treatment, and corresponding (equivalent) images of an age-matched untreated patient with LI-MLD (in parenthesis, the chronological age at imaging acquisition in months). In MLD01 images, a small area of hyperintensity is present within the splenium of the corpus callosum, stable in extension and appearance as compared with that in the +12 months follow-up; subtle signal inhomogeneities are present within posterior periventricular and posterior centrum semiovale white matter, in the absence of focal lesions; these inhomogeneities, present since baseline as just barely T2 and FLAIR hyperintensity signal, are (now) more evident because of the normal signal of the surrounding myelin; subarachnoid and ventricular spaces are within normal limits, even if a little wider when compared with that of the baseline. Basal ganglia and thalami remain of normal appearance. In UT LI-MLD images, extensive, diffuse symmetric hyperintensities with typical "tigroid pattern" are seen within periventricular white matter, centrum semiovale, corpus callosum, external and internal capsules, and cerebellar deep white matter. A severe diffuse brain atrophy involving basal ganglia and thalamy, which show T2 hypointense signal, is also present.

abundant IS-marked cell clones at any given time point of the follow-up. IS targeting *OPTC* were not detected in samples from the longest follow-up time point.

Extensive HSC Gene Marking in Vivo

To assess HSC gene marking in vivo, we analyzed the subset of IS shared among three data sets representing progenitors and mature myeloid and mature lymphoid cells in each patient. After stringent filtering to reduce the false discovery rate due to the impurity of each cell fraction analyzed and the occurrence of collisions during IS processing (fig. S20 and table S17), a fraction of ISs were consistently shared among the three data sets of each patient (Fig. 6A). This fraction increased with follow-up time (Fig. 6B). We then plotted the occurrence of shared ISs in each subset of cells over time, considering the B and T cells separately, and ranked them for multiple hits (Fig. 6C and table S18). The increasing presence over time of $CD34^+$ progenitors and mature cells of myeloid and lymphoid lineages marked by identical integrations is evidence of self-renewal

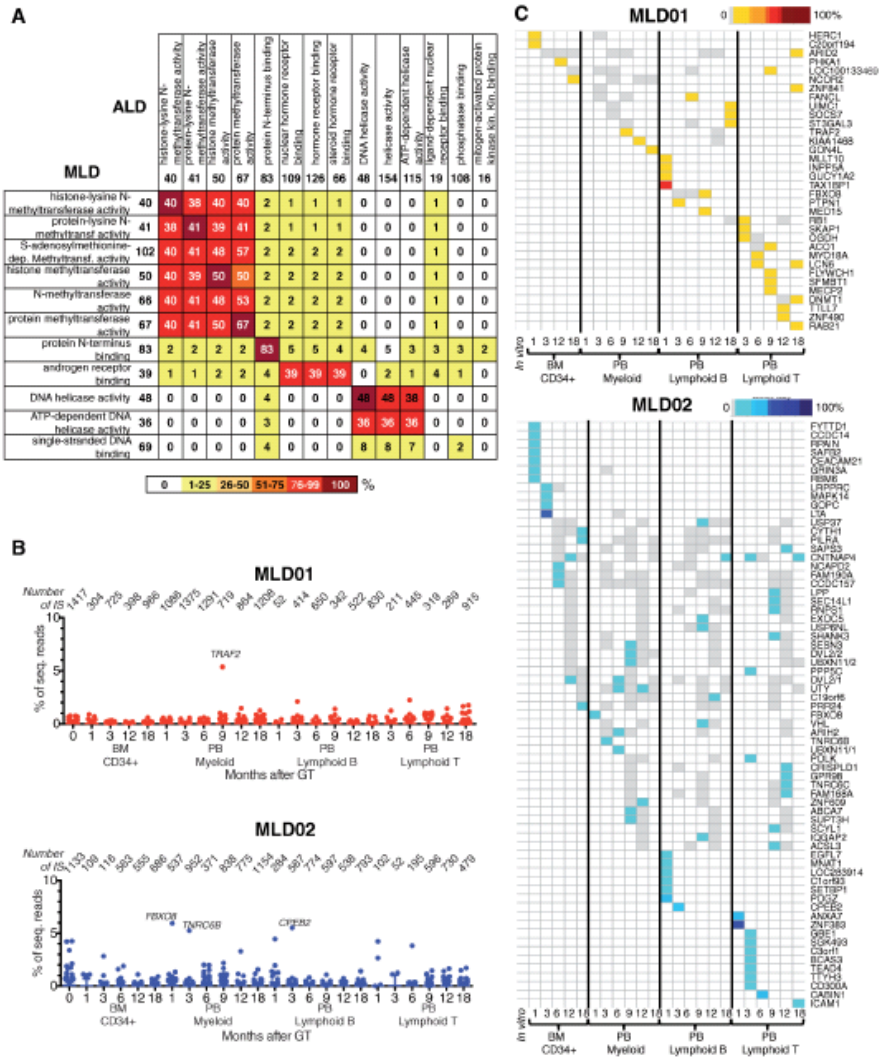


Fig. 4. LV genomic integration profile. (**A**) Gene ontology (GO) analysis on ISs from three patients with MLD and two patients with ALD who had been treated with HSC-GT (*17*). This analysis was performed using the GREAT software (http://bejerano.stanford.edu/great/). The genomic positions of ISs in each data set were weighted by the binomial test in order to evaluate the clustering at given genomic regions and by the hypergeometric test in order to evaluate the overrepresentation of gene functions. GO classes were considered significant when both tests provided a false discovery rate of <0.05. To compare the level of similarity between the gene classes preferentially targeted by LV integrations in ALD and MLD clinical trials, we counted the number of shared genes contained in the significantly overrepresented gene classes for GO molecular functions of each clinical trial (results for GO biological processes are shown in fig. S13). The extent of gene sharing between the overrepresented gene classes are indicated by a color scale. (**B**) Box plot of the percentage of sequence reads (*y* axis) for unequivocally mapped ISs from patients MLD01 and MLD02 in different cell types ($CD34^+$, myeloid, or lymphoid cells) from different sources (BM or PB) and time points (months after gene therapy, indicated below the *x* axis). The number of reads for each IS was normalized to the total number of sequence reads from the same time point and source. ISs over the 95 percentile of the data set are shown as dots distinct from the box and whiskers, which are mostly flattened to the bottom of the plot. The total number of ISs for each lineage and time point is shown on top. Most represented integrations (with the hit gene indicated next to the dot) are enriched in oligoclonal populations, such as in PB-derived B or T cells at early time points. (**C**) Follow-up of the most represented ISs during time in different cell types. Percentages of sequence reads for each IS are calculated on the total number of sequence reads from the same lineage so as to emphasize the maximum relative contribution, thus obtaining values that are greater or equal to percentages shown in (B). For each IS, colored cells indicate retrieval at ≥5%, with higher color intensity indicating higher percentage, whereas gray cells indicate retrieval at low percentage (from 0.006% to <5%). Lack of color indicates that the integration was not retrieved at the indicated time point and source. The targeted genes are indicated. Almost all integrations reached ≥5% at only one time point.

and multilineage potential of the transduced engrafted HSCs.

To estimate the number of HSCs contributing to hematopoietic reconstitution, we used the fraction of ISs shared between independent samplings of the same cell population (supplementary materials, materials and methods). For this analysis, we considered the output of short-lived mature myeloid cells in the PB long-term after HSC-GT as readout of total HSC activity at that time. We compared ISs shared between $CD14^+$ and $CD15^+$ PB cells at months 9, 12, and 18 after HSC-GT by the mark-recapture approach using the Petersen/Schnabel estimator method (*31*) and Chao Poisson regression model (table S19). The resulting lower bound population size estimates were 3.7×10^3 to 5.7×10^3 in patient MLD01 and 2.1×10^3 to 3.4×10^3 in MLD02, indicating an abundant pool of engrafted transduced self-renewing progenitors in both patients.

To quantify clonal diversity in each lineage over time, we calculated the Shannon Diversity Index (Fig. 6D and fig. S22). This index measures the entropy of an IS data set taking into account the total number of ISs and their relative contribution. The diversity of $CD34^+$ progenitors was lower early after HSC-GT as compared with that of the infused cell population and then increased and stabilized. PB myeloid cells showed high and stable diversity, which is consistent with that of the progenitor pool, whereas B and T cell diversity increased with time and stabilized 6 months after gene therapy. Patient MLD03 showed lower IS sharing between lineages and decreasing diversity of myeloid cells as compared with the other two patients, possibly because of the shorter follow-up and the higher VCN of the infused cells.

Overall, these data provide evidence that efficient ex vivo gene transfer was followed by substantial engraftment and sustained clonogenic activity of the transduced HSCs in the patients, resulting in extensive polyclonal reconstitution of hematopoiesis with gene-corrected cells.

Discussion

LI MLD is a devastating disease that invariably leads to death of affected children within a few years of symptom onset. We have shown that HSC-GT halted disease manifestation and/or progression in three presymptomatic children with LI-MLD for follow-up times ranging from 18 to 24 months after treatment (7 to 21 months after predicted disease onset).

The clinical benefit observed in the three patients was accompanied by measurable objective findings ascertained through brain imaging and electrophysiological and biochemical studies and is in contrast with the disease evolution observed in (i) the patients' older affected siblings with matched ARSA mutations and related genetic background and (ii) our historical cohort of untreated LI-MLD patients. MRI analysis detected a minor demyelinating lesion in the brain of MLD01 1 year after treatment. This minor lesion, which might have arisen before the infused cells reached a sufficient CNS engraftment to exert beneficial effects, remained stable at all subsequent follow-ups. This finding is clearly different from what is observed in untreated LI-MLD patients, who show rapidly progressive and widespread brain demyelination. The severe peripheral neuropathy already present in MLD01 at the time of HSC-GT improved after treatment, with amelioration of the amplitude of motor action potentials and of the nerve conduction velocities after treatment. These findings were associated with clinical evidence of normal cognitive evolution and continuous improvements in motor skills. Although the follow-up is shorter for MLD02 and MLD03, these patients remained asymptomatic at a chronological age when their disease was expected to have manifested.

In previous work with the MLD mouse model, we showed that the therapeutic benefit of

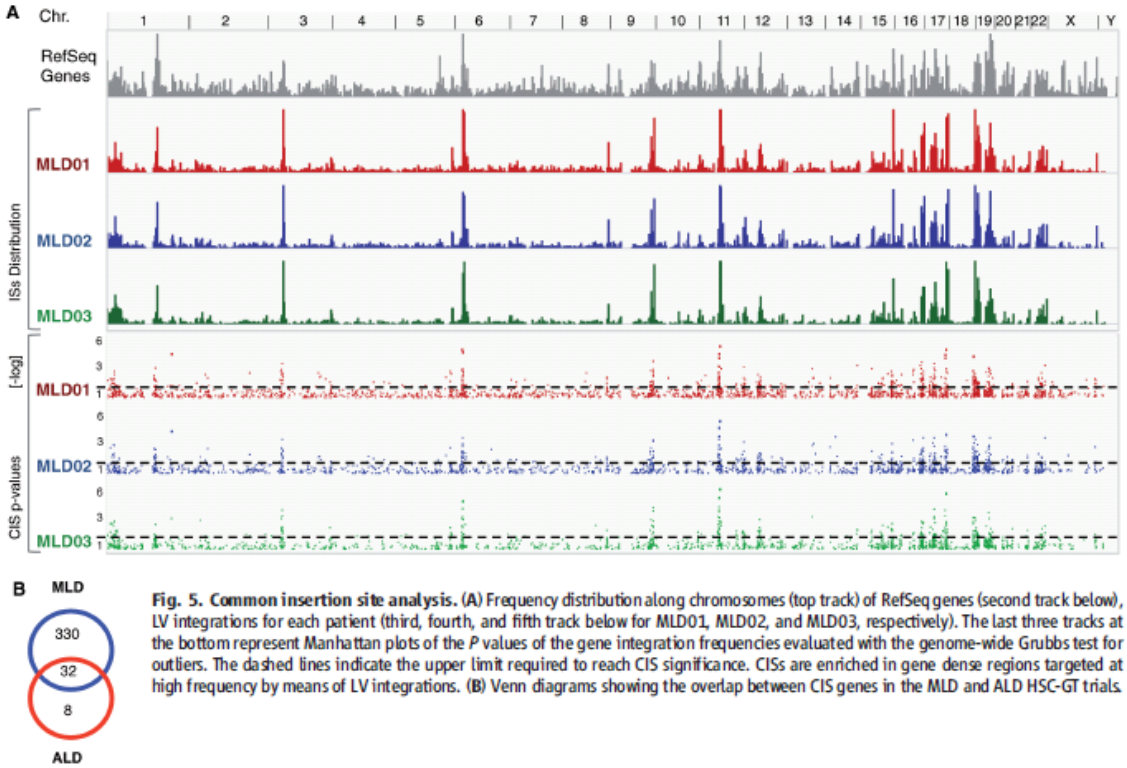


Fig. 5. Common insertion site analysis. (**A**) Frequency distribution along chromosomes (top track) of RefSeq genes (second track below), LV integrations for each patient (third, fourth, and fifth track below for MLD01, MLD02, and MLD03, respectively). The last three tracks at the bottom represent Manhattan plots of the *P* values of the gene integration frequencies evaluated with the genome-wide Grubbs test for outliers. The dashed lines indicate the upper limit required to reach CIS significance. CISs are enriched in gene dense regions targeted at high frequency by means of LV integrations. (**B**) Venn diagrams showing the overlap between CIS genes in the MLD and ALD HSC-GT trials.

HSC-GT arises from the homing of ARSA-overexpressing HSPCs and/or their myeloid progeny to the CNS and PNS, where they contribute to the replacement of a substantial fraction of microglia/resident macrophages, scavenge the stored sulfatide, and establish a local source of functional enzyme bioavailable throughout the neural tissues. Although we could not directly probe our patients for the migration of gene-modified cells into the CNS, we documented that CSF samples from the patients contained substantial levels of ARSA protein and activity long after HSC-GT. This objective finding, together with the halted progression of neural disease, suggests that the CNS and PNS were seeded by gene-corrected myeloid cells upon HSC-GT.

Recent studies in mice have shown that microglia mostly originates from an early seeding of neural tissues by embryonic lineage macrophages followed by in situ turnover under homeostatic conditions (*32*). However, substantial replacement of microglia with donor-derived cells was shown after myeloablation and intravenous infusion of HSPCs (*8*, *33*, *34*). The latter findings are consistent with clinical evidence that HSCT can provide therapeutic benefit in certain lysosomal storage diseases affecting the CNS, and with the few available studies investigating the donor origin of microglia cells within the brains of transplanted humans (*35*, *36*). We speculate that high-level engraftment of the myeloid compartment in PB and BM by the transduced HSPCs is likely needed to support their efficient migration to the neural tissues. It is possible, however, that myeloid progenitors, rather than mature monocytes, are primarily responsible for seeding the CNS in our patients (*34*).

At least three factors may have contributed to the high levels of gene replacement observed in patients' hematopoiesis. First, we optimized the LV-manufacturing process, aiming to produce vector with high titer, infectivity, and purity using stringent lot-release criteria so as to ensure efficient transduction while limiting detrimental effects on HSPCs. Second, we validated the transduction protocol for reproducible high-rate gene transfer into BM-derived mouse-repopulating human HSPCs and infused fresh cells after transduction to avoid incurring in any cell loss associated with freeze/thawing. Last, we administered a dose-adjusted myeloablative regimen before HSPC infusion to foster engraftment.

Another key goal of our study was to assess the long-term safety of HSC gene transfer. Using deep-sequencing and bioinformatics, we found no evidence that cell clones harboring ISs undergo in vivo expansion or selection. Rather, our data suggest that the genomic distribution of IS in patients' hematopoietic cells reflects the LV integration in the infused HSPCs. These findings are in sharp contrast with those reported at comparable times after HSC-GT in some other trials using γ-RV; in those trials, cell clones harboring

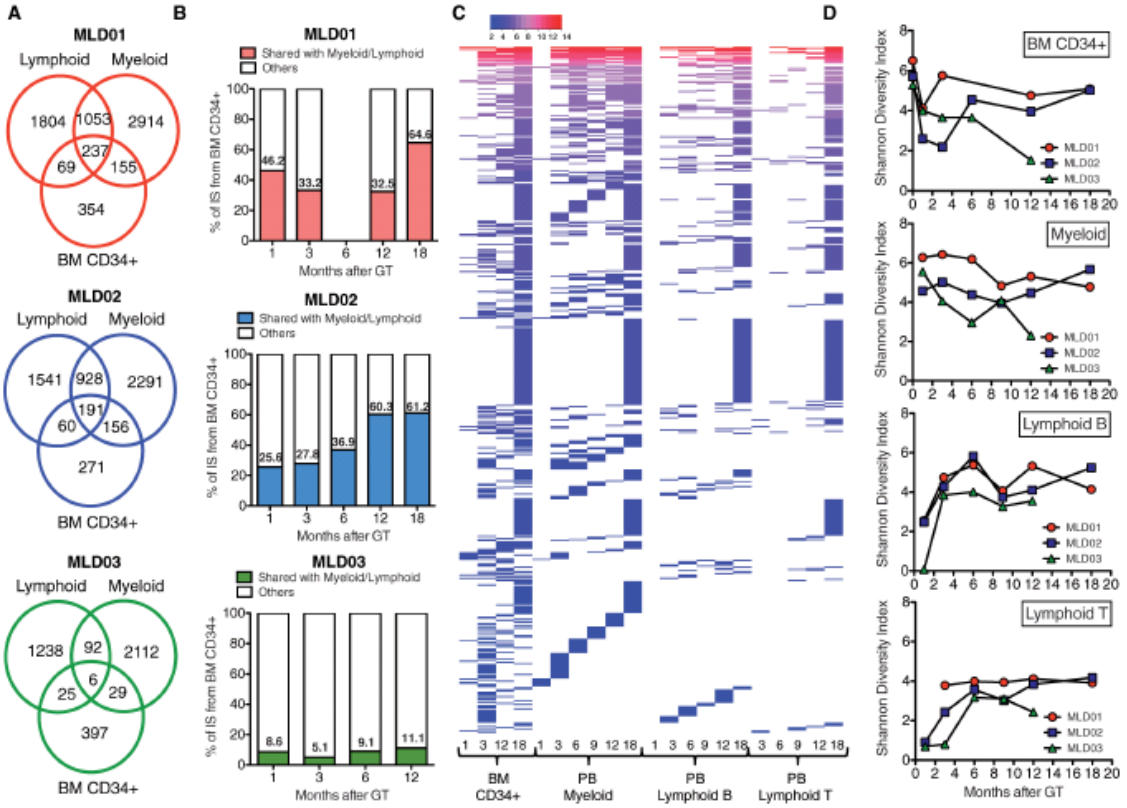


Fig. 6. Stem cell marking and clonal dynamics. Analysis of IS data was performed after filtering for collision and sample impurity. (**A**) Venn diagrams showing the sharing of ISs between CD34$^+$ cells and lymphoid and myeloid lineages. (**B**) Percentage of shared ISs (*y* axis) between BM-derived CD34$^+$ and myeloid or lymphoid lineages during time (months after GT, indicated below). (**C**) Tracking of ISs shared between multiple lineages with time in patient MLD01. Each row represents a specific IS, with colored bars indicating retrieval from the indicated cell lineage and time point after gene therapy (columns). The line color varies with the degree of sharing among lineages (red, high level of sharing; blue, low level of sharing; white, no integration retrieved). PB, peripheral blood; B, B cells; T, T cells; Myelo, myeloid cells. (**D**) Shannon Diversity Index (*y* axis) was calculated to measure the clonal diversity of hematopoietic reconstitution during time.

ISs that target oncogenes underwent expansion or selection in vivo and eventually progressed to leukemia (*14*, *18*, *37*–*40*). The LV IS distribution in MLD patients overlaps with that reported for ALD patients after LV-based HSC-GT, a reassuring finding considering that no adverse effect of gene transfer has been reported in that trial after more than 5 years of follow-up. A similar integration profile in the absence of clonal expansion was observed in three patients with Wiskott-Aldrich Syndrome treated by LV-based HSC-GT with a follow-up of up to 2.5 years (*47*). Overall, these data strongly support the improved safety of LV comprising self-inactivating long terminal repeat and moderately active internal promoters, which makes it unlikely that an insertion would activate a nearby oncogene (*19*, *20*, *41*). Nevertheless, we will continue longer-term evaluation of the treated and additional patients for full assessment of the risk-benefit ratio and therapeutic potential of HSC-GT.

The stability of gene marking together with the identification of several ISs shared among progenitors, mature myeloid cells, and both major lymphoid lineages long-term after HSC-GT provide evidence that bona fide HSCs were transduced. Furthermore, the estimated large number of active HSCs would imply that a good fraction of harvested HSCs were transduced and long-term engrafted the patients. These data position LV gene transfer as a feasible means to engineer human hematopoiesis to its near entirety, an approach that may be exploited to develop novel treatments for several inherited and acquired diseases.

Materials and Methods

Clinical Protocol and Patients

The Istituto Superiore di Sanità and the Ethical Committee of the San Raffaele Scientific Institute in Milan approved the study. The study promoter is TIGET, San Raffaele Hospital, Italy and the financial sponsor of the study is the Telethon Foundation. The medicinal product received Orphan Drug Designation (ODD) (EMEA/OD/102/06) by the European Medicines Agency (EMA) for the treatment of MLD.

We enrolled and treated three presymptomatic LI-MLD patients, who were classified as LI according to the disease in their affected older siblings using the historical classification based on age at symptom onset (table S3). The patients were from Lebanon, the United States, and Egypt. Upon obtaining informed consent, their diagnosis was molecularly and biochemically confirmed. In all patients, the definitive MLD diagnosis was based on pathologic low ARSA activity and the presence of disease-causing *ARSA* gene mutations on both alleles. Presymptomatic condition at enrollment was defined on the basis of a normal neurological and cognitive examination, independently from the presence of abnormalities at neurophysiological tests or brain imaging.

Details on clinical study enrollment criteria, endpoints, and study plan can be found at ClinicalTrials.gov #NCT01560182. The parents of all subjects provided written informed consent for experimental treatment. Biological samples were obtained from MLD patients—healthy children and adults as controls—with approval of the San Raffaele Scientific Institute's Ethics Committee and consent from parents or subjects.

Neurological and Motor Evaluation

A standard neurological evaluation was performed in all patients for assessing the presence of disease clinical signs. In addition, we applied GMFM (*26*, *42*) and the Gross Motor Function Classification for MLD (GMFC-MLD) (*27*) for proper quantification of patients' motor abilities. The same trained personnel applied the scales at each evaluation.

Neuropsychological Evaluation

Neuropsychological evaluation was performed by using the Bayley Scale for Infant and Toddler Development (BSID-II) (*43*), applied at baseline evaluation and then every 6 months.

Neurophysiological Evaluation

Sensory conduction of sural and median nerves, and motor conduction of deep peroneal and ulnar nerves were studied and a *z* score for NCV was calculated for each examined nerve (patient's NCV – normal donors' mean NCV/normal donors' NCV standard deviation); an NCV index was calculated as the average of *z* scores obtained from the four tested nerves for each patient per time, as described (*26*).

MR Examination

All children underwent anatomical MRI on a 1.5 Tesla scanner using a six-channel SENSE head coil (Gyroscan Intera, Philips, Netherlands). MRI scans were performed with Spin Echo T1 weighted images, repetition time (TR) 600 ms, echo time (TE) 15 ms, rec matrix 288, 22 slices, 5 and 3 mm thick, axial and sagittal planes; Turbo Spin Echo T2-weighted images, TE120 ms, TR 5500 ms 24 slices, axial, sagittal and coronal planes, 4.5 and 3 mm thick; fluid-attenuated inversion recovery Turbo Spin Echo, TE 140 ms, 24 slices, 4.5 mm thick; and volumetric T1 sequence (fast field echo, 110 slices, 1.4 mm thick, and 0 mm gap). MR examinations were performed under sedation by a senior experienced pediatric anesthesiologist (*44*); sedation was induced with intravenous 1 mg/kg propofol and maintained with 4 mg/kg/hour propofol continuous infusion. Throughout MRI examination, saturation of peripheral oxygen (SpO_2) was monitored with a MRI-compatible device (Magnitude, Invivo Corporation, Orlando, Florida). Desaturation (SpO_2 < 95%) was recorded together with any adverse event requiring the intervention of the attending anesthesiologist.

Large-Scale Lentiviral Vector Production

The vector used in this study (pCCLsin.cPPT.hPGK.hARSA.WPREmut6 - PGK.ARSA.LV) is a self-inactivating lentiviral vector produced with a third-generation split packaging system and pseudotyped by the vesicular stomatitis virus G protein (VSV.G) (*6*). The vector lots were produced by MolMed (Milan, Italy) using a large-scale validated process. The vectors were produced following Good Manufacturing Practice (GMP) guidelines, except for initial studies of gene transfer protocol optimization, which used pre-GMP (GMP-like/large scale produced) vector. The protocol for vector production is detailed in fig. S3. Briefly, the ARSA LV was produced by means of transient four-plasmid transfection of 293T cells. 293T cells were derived from human embryonic kidney 293 cells with stable transfection of the temperature-sensitive SV40 T-antigen; a subclone of the 293T (from Stanford University) selected at the Salk Institute (La Jolla, California) for its high-yield performance in production of lentiviral vectors through transient transfection was used for MCB establishment; a 293T MCB was generated at Genethon (Paris, France) in GMP conditions. 293T cells from the master cell bank were expanded in T162 flasks and then in 10-tray cell factories and transiently transfected by means of calcium phosphate precipitation with four plasmids encoding for two core packaging constructs (pKLGag/pol and pKRev), the envelope construct (pK.G), and the transfer vector construct (pARSA). Twenty-four hours after removal of the transfection medium, the cell supernatant was harvested and stored at 4°C. The culture medium was replaced, and after a further 24 hours, a second harvest was performed. The medium collected from the two harvests was pooled and filtered through 5/0.45-μm filters so as to discard cell debris. The downstream purification process included a benzonase treatment overnight at 4°C, followed by a diethylamino anion exchange (DEAE) chromatography step, concentration, and gel filtration in CellGro medium. The benzonase treatment was aimed at degrading plasmid DNA and host cell–derived DNA present in the clarified cell supernatant, thus facilitating their removal through subsequent purification steps. Anion exchange chromatography involved the absorption of negatively charged lentiviral particles to positively charged chromatographic support; viral particles were eluted by increasing ionic strength. This step was aimed at the removal of contaminants such as host cell proteins and serum-derived proteins such as bovine serum albumin (BSA). Gel filtration, also defined as size exclusion chromatography, relies on the inability of the large lentiviral particles to be retained by matrix pores. This step was aimed at the removal of salts and small-sized contaminants such as DNA fragments as well as at medium exchange. The resulting LV preparation, in CellGro medium, underwent one sterilizing 0.2-μm filtration and aseptic filling. The purified vector preparation was stored at –80°C. The produced vector lots were characterized as described in table S2. Physical titer was measured by means of enzyme-linked immunosorbent assay (Perkin Elmer, Applied Biosystem, Foster City,

California) for the detection of HIV-1 gag p24 capsid protein. Infectious titer was measured through transduction of a human T cell line with serial dilutions of vector and calculation of the copies of integrated vector per cell by quantitative PCR.

HSC Isolation and Transduction

For in Vitro Experiments

Healthy donor (HD) bone marrow $CD34^+$ cells were purchased from Lonza (Basel, Switzerland) or obtained after written informed consent according to standard ethical procedure and with approval of the San Raffaele Institute Ethical Committee (protocol TIGET-01). $CD34^+$ cells from three MLD patients not enrolled in the gene therapy trial—diagnosed on the basis of clinical symptoms, ARSA activity, and genetic analysis—were similarly obtained after written informed consent (protocol LDM1) at the time of diagnostic or therapeutic procedures. $CD34^+$ cells were purified from mononuclear cells by means of positive selection with immunomagnetic beads according to the manufacturer's procedure (Miltenyi Biotec, Bergisch-Gladbach, Germany). Soon after purification or thawing, $CD34^+$ cells were placed in culture on retronectin-coated non–tissue culture–treated wells (T100A Takara) in CellGro SCGM medium (2001 CellGenix) at a concentration of 1×10^6 cells/ml in the presence of cytokines [interleukin-3 (IL-3, 60 ng/ml), thrombopoietin (TPO, 100 ng/ml), stem cell factor (SCF, 300 ng/ml), and Flt3 ligand (Flt3-L, 300 ng/ml); PeproTech, Rocky Hill, New Jersey] for 24 hours of prestimulation. Cells were then transduced with ARSA LV [at a multiplicity of infection (MOI) of 100] for 14 hours. In the protocol that foresees two rounds of transduction, selected for clinical application, cells were washed for 10 hours in CellGro SCGM medium additioned with cytokines and underwent a second hit of transduction in the same conditions as the first. At the end of transduction, cells were counted and collected for clonogenic assays, flow cytometry, and in vivo studies. Remaining cells were plated in Iscove's modified Dulbecco's medium (IMDM) –10% fetal bovine serum (FBS) with cytokines (IL-3, 60 ng/ml; IL-6, 60 ng/ml; SCF, 300 ng/ml) and cultured for a total of 14 days. Thereafter, cells were collected for molecular and biochemical studies.

For Patients' Treatment

Please refer to fig. S2. Total bone marrow was collected from the iliac crests under sterile conditions and using general anesthesia, on day –4, according to an internal standard operating procedure. The total collected volume was estimated according to the content of $CD34^+$ cells evaluated by previous bone marrow aspiration; on average, 20 to 25 ml/kg patient's body weight was collected. The harvested bone marrow—collected in a dedicated bag, sealed, and identified—was then transferred to MolMed S.p.A and kept in sterile conditions in a refrigerator at 4°C for 24 hours. $CD34^+$ cells were then purified on day –3 from mononuclear cells by means of positive selection with immunomagnetic beads according to the manufacturer's procedure (Miltenyi Biotec, Bergisch-Gladbach, Germany) in GMP conditions. The purified cells were cultured in retronectin-coated VueLife bags (American Fluoroseal, Gaithersburg, Maryland) in serum-free medium supplemented with GMP-grade cytokines and exposed twice to GMP-grade–purified vector at multiplicity of infection of 100, for a total culture time of 60 hours. At the end of the transduction procedure, the transduced $CD34^+$ cells were harvested, washed with Cell Grow medium, and resuspended in saline solution at the concentration of 2×10^6 to 10×10^6/ml for patient's infusion. At the end of transduction, a fraction of the cells was collected for clonogenic assays, flow cytometry, and in vitro culture, as above. Cells were kept at 4°C up to the time of infusion, upon batch release according to quality control tests [results are available at infusion for viability, immunophenotype, endotoxin, large T Ag DNA, and mycoplasma (table S4)].

Patients' Conditioning

Patients received a total of 14 body weight–based doses of intravenous busulfan, given every 6 hours from day –4 to day –1. The busulfan plasmatic levels were monitored with serial sampling following first and fifth infusion. The doses after the 5th and 10th were adjusted, if needed, according to plasmatic Busulfan levels with an ideal AUC of range 4200-5600 μg/L*hour, target 4800 μg/L*hour. The interval between the end of the last intravenous busulfan dose and final product infusion was ≥24 hours in order to allow safe infusion of the stem cells. At day 0, after premedication with clorfeniramine (0.25 mg/kg, max dose 10 mg) or equivalent drug, the resuspended $CD34^+$ cell product was infused intravenously through the central catheter.

During hospitalization, patients were cared for in an isolation unit and received supportive therapy according to local standards.

Isolation of Hematopoietic Subpopulations from Patients' Peripheral Blood and Bone Marrow

Starting from mononuclear cells (MNCs) obtained from Lymphoprep gradient, human bone marrow and peripheral blood subpopulations were obtained through positive selection of the following markers (Miltenyi Biotec microbeads): CD34, CD13, CD15, CD56, CD19, CD3, glycophorin A (GLYA), and CD61 from the BM, and CD15, CD14, CD56, CD19, and CD3 from PB, according to manufacturer's procedures. Purity of each subpopulation was checked by means of flow-cytometry. Data are reported in table S11.

Clonogenic Assay (CFC)

The CFC assay was performed by means of plating in methylcellulose medium (human MethoCult; StemCell Technologies) either 800 to 1000 $CD34^+$ cells/ml or 1.25×10^4 to 1×10^6 MNCs/ml purified from BM or PB. After 14 days of culture, colonies were counted, distinguishing myeloid and erythroid origin, and plucked for molecular analysis.

Quantitative PCR

Genomic DNA was extracted from $CD34^+$ liquid culture, peripheral blood mononuclear cells (PBMCs), MNCs, PB, and BM subpopulations of MLD patient samples with QIAamp DNA Blood Micro and Mini Kits (Qiagen, Hilden, Germany). Single colonies plucked from the CFC assay were digested for 4 hours at 37°C in Lysis Buffer (Lauryl Ether 0.1%, TRIS/hydrochloride (HCL) 10 mM, 100 μg/ml Proteinase K). After proteinase K inactivation (10 min at 95°C), lysates were centrifuged at 13,000 revolutions per min at 4°C for 15 min.

LV sequences were detected by means of quantitative PCR on 25 to 50 ng of total genomic DNA or 10 μl of lysate supernatants by using previously described primers and probe (*22*).

Absolute quantifications were plotted on standard curves prepared with serial dilutions of genomic DNA or cell lysate from a T cell clone containing 1 copy HIV/cell.

Engraftment of the transduced cells was evaluated by means of quantitative PCR on individual colonies from CFC assay performed on either PB- or BM-derived cells and expressed as percentage (%) of LV^+ (VCN ≥ 0.5) colonies on total tested colonies (≥ 250 cells/colony).

Analysis of Vbeta Repertoire

The analysis of Vbeta repertoire of the T cell receptor was performed on 50 μl of patients' total PB according to the manufacturing procedure described in the V Beta kit; Beckman Coulter (823497) (Fullerton, California). 10000 events were acquired with FACS Canto II (BD-Biosciences, Europe) and then analyzed with FlowJo (TreeStar, Ashland, Oregon). The percentage of each Vbeta population was compared with the range of expression obtained from the analysis of a pool of 85 healthy donors provided by the manufacturer.

Immunophenotype Analysis

Analysis of the immunophenotype of patients' total PB and BM was performed by Laboraf (Ospedale San Raffaele) as described by internal SOPs.

ARSA Activity Determination

p-Nitrocatechol Sulfate (PNCS)

$CD34^+$ liquid culture, PBMCs, MNCs, PB, and BM subpopulations of MLD patients and healthy donors were resuspended in sodium acetate trihydrate 0.05 M (500,000 cells in 25 μl). Samples were then sonicated and centrifuged, and the supernatant was collected for ARSA activity and protein quantification (Bradford assay; Biorad #500-0006) (Hercules, California). ARSA activity was detected with PNCS as substrate, as described, loading a maximum concentration of

protein extract of 0.3 μg/μl diluted in sodium acetate trihydrate 0.05 M (9).

DEAE-Cellulose Chromatography and 4-Methyl-Umbellipheryl-Sulfate

Cells were harvested, washed in phosphate-buffered saline, and resuspended in 10 mM TRIS/HCl buffer, pH 7.5, containing 0.1% (v/v) Nonidet NP40 detergent (Sigma-Aldrich, St. Louis, Missouri). Cell lysates were subjected to three rounds of sonication. Procedures were carried out at 4°C. Proteins were measured with the Bradford method by using bovine serum albumin as standard.

Similar cell number (500,000 cells) was loaded into a 0.2-ml DEAE chromatography column equilibrated with a 25 mM TRIS/HCl buffer, pH 7.5. The flow rate was 0.2 ml/min. After washing with 5 ml of 25 mM TRIS/HCl buffer plus 50 mM NaCl, pH 7.5, enzyme activity retained by the column was eluted by 2.5 ml TRIS/HCl buffer plus 250 mM NaCl, pH 7.5 (starting from fraction n.5). Fractions (0.5 ml) were collected and assayed for the ARSA activity by using the artificial fluorogenic substrate 4-methyl-umbellipheryl-sulfate (MUS) dissolved in 0.05 M Na-acetate/acetic acid buffer, pH 5.5. A volume of 50 μl of test sample was incubated with 100 μL of a 0.01 M solution of substrate respectively in the presence and absence of 125 μM AgNO3 (a specific ARSA inhibitor) at 37°C. Enzymatic reactions were stopped by adding 0.2 M Glycine/NaOH, pH 10.6. Fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin Elmer LS3 spectrofluorimeter (excitation 360 nm, emission 446 nm). ARSA activity was calculated by subtracting the value obtained in the presence of AgNO3 [arylsulfatase B (ARSB) activity] from that measured in the absence of the inhibitor (ARSA+ARSB activity) (*45*).

Sulphatide Assay

Sulfatide (5 mM) (Matreya, St. Pleasant Gap, Pennsylvania) was resuspended in cloroform: methanol:water (2:1:0.1), dried overnight at room temperature, and resuspended in the ARSA enzymatic buffer [100 mM Na/acetate, pH 4.5, containing 20 mM MnCl2 and taurodeoxyxholate (100 mg/50 μl)]. For the reaction (100 μl total volume), 0.003 mU ARSA were added to 21 nmol sulfatide at 37°C for 24 hours. The assay was stopped with 0.85 ml cloroform:methanol:water (1:1) plus 0.85 50 mN sulfuric acid and 0.170 ml Azure A [0.4 mg/ml]. After a centrifugation at 300 g × 5 min, the lower phase was recovered, and the absorbance measured at 650 nm in a microplate reader (GDV DV-990BV6). The nanomoles of sulfatide hydrolyzed by ARSA were calculated by subtracting the value measured in the presence of the enzyme respect to a reference assay performed in the absence of ARSA.

In Situ Hybridization and Immunofluorescence

Cells were grown on polyornithine-coated glass coverslips (0.01% solution, Sigma-Aldrich Corp, Italy) 72 hours in IMDM 10% FBS medium and then fixed with 4% paraformaldehyde (PFA) solution.

An antisense 598–base pair digoxigenin-labeled oligonucleotide complementary to W-Pre mRNA sequence and a control sense sequence were produced .The in situ protocol was modified from (*46*). Cells were treated with prehybridization protocol and incubated overnight at 37°C with 0.5 μg/ml probe. The probe was detected by sheep anti Digoxigenin 1:500 (Roche Applied Science, Penzberg, Germany) overnight 4°C.

Immunofluorescence on ARSA-hemagglutinin (HA) (9)–transduced Hela cells and on patients' and healthy donors' PB cells or HeLa cells was performed overnight at 4°C by using rabbit anti–Human Lamp1 1:100 (Abcam), goat anti–Human ARSA 1:50 (Abnova), and/or rat anti-HA 1:100 (Roche Applied Science). Secondary antibodies were incubated 1:500 for 1 hour at RT as follows: donkey anti–sheep Alexa488, donkey anti–goat Alexa546, donkey anti–rat Alexa488, and donkey anti–rabbit Alexa647. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Images were acquired with Leica (Solms, Germany) TCS SP2 laser scanning confocal with 63× oil objective.

Rag2$^{-/-}$ Il2rγ$^{-/-}$ Mice Transplantation and Engraftment Evaluation

Rag2$^{-/-}$ Il2rγ$^{-/-}$ mice were obtained from the Central Institute for Experimental Animals, Nogawa, Japan and maintained in our animal facility according to approved protocols. Three-day-old mice were sublethally irradiated (40,000 μGy/m^2) 24 hours before intravenous injection of untransduced and unmanipulated or transduced CD34$^+$ cells. Eight to 12 weeks after the transplantation, mice were killed and hematopoietic organs (bone marrow, spleen, and thymus) were collected after intracardiac PBS perfusion and processed for fluorescence-activated cell sorting (FACS) analysis. All procedures were performed according to protocols approved by the Animal Care and Use Committee of the San Raffaele Scientific Institute (IACUC #325) and communicated to the Ministry of Health and local authorities according to Italian law.

Flow Cytometry

Patients' samples—subpopulation analysis

From each sample, 5000 cells were washed and resuspended in blocking buffer (PBS with 5% FBS 2% BSA Fc Block 1:100), then 1 μl of the following human-specific conjugated antibodies were added: APC-conjugated anti-CD56, PE-conjugated anti-CD34, anti-CD13 and anti-glycophorinA, TC-conjugated anti-CD19, and anti-CD3, FITC-conjugated anti-CD15 and anti-CD61, PB-conjugated anti-CD14 under unstained control (DAKO, Denmark; BD-Pharmigen, Europe).

Rag2$^{-/-}$ IL2rγ$^{-/-}$ mice—engraftment evaluation

From each sample, 250,000 cells were washed and resuspended in blocking buffer (PBS with 5% FBS 2% BSA Fc Block 1:100), then 0.5 μl of the following human-specific conjugated antibodies were added: APC-conjugated anti-CD45, PE-conjugated anti-CD34, anti-CD13, anti-CD4, anti-CD11b and TC-conjugated anti-CD19, anti-CD8, anti-CD3 under control of relative IgG isotype control (DAKO; BD-Biosciences).

After 20 min on ice, cells were washed, resuspended in PBS 2% FBS, 1% PFA for cytometric analysis. Of 100,000 events, 3000 were acquired with FACS Canto II (BD-Biosciences, Europe) and then analyzed by FlowJo (TreeStar, Ashland, Oregon). During quadrant analysis, only fluorescence that excluded >99.9% of isotype control events was considered to be specific.

Vector Integration Site Analysis

A detailed description of the materials and methods used for vector integration site analysis is reported in the supplementary materials, materials and methods section.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1233158/DC1
Materials and Methods
Figs. S1 to S22
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Chapter 7

"Case series, clinical reports and invited commentary on the use of new drugs in pediatric population, characterization of severe and unusual conditions and description of the State of the Art of known patologies"

ORIGINAL ARTICLE: GASTROENTEROLOGY

Successful Use of Long-Acting Octreotide for Intractable Chronic Gastrointestinal Bleeding in Children

†Marie O'Meara, ||Maria P. Cicalese, ‡Andrea Bordugo, §Alessandro Ambrosi, *Nedim Hadzic, and *Giorgina Mieli-Vergani

ABSTRACT

Background and Aims: Octreotide reduces splanchnic blood flow and is effective in controlling gastrointestinal bleeding (GIB) caused by portal hypertension. Monthly long-acting octreotide (OCT-LAR) with an efficacy and safety profile similar to subcutaneous daily administration presents an attractive option for long-term therapy. We report our experience with OCT-LAR for severe/recurrent GIB in children with portal hypertension secondary to chronic liver disease or portal vein thrombosis who were unresponsive to standard interventions.

Methods: A total of 9 patients, 7 boys, who received OCT-LAR between 2000 and 2009 were studied retrospectively (median age at first bleeding 21 months, range 1 month–14.5 years). The dose (2.5–20 mg intramuscularly monthly) was extrapolated from that used in adult acromegaly and neuroendocrine tumours (10–60 mg/mo). Response to treatment was assessed by comparing the number of bleeding events, hospital admissions for acute bleeding, and number of blood units required during the year before and year after starting OCT-LAR.

Results: OCT-LAR led to a reduction in the number of bleeding episodes in all of the children and to cessation of bleeding in 7. Two children listed for transplantation because of severe GIB were removed from the list. No serious adverse effects immediately attributable to OCT-LAR were observed. One child developed growth hormone deficiency and hypothyroidism during a prolonged period of treatment with subcutaneous octreotide before commencing OCT-LAR.

Conclusions: OCT-LAR can control severe intractable recurrent GIB in children with portal hypertension. Prospective randomised controlled trials and pharmacokinetic studies are indicated to establish the optimum dose and length of treatment of OCT-LAR and confirm its efficacy and long-term safety in children.

Key Words: children, depot octreotide, gastrointestinal bleeding, long-acting octreotide, octreotide, portal hypertension

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From the *Paediatric Liver, GI and Nutrition Centre, the †Department of Pharmacy, King's College Hospital NHS Foundation Trust, London, UK, the ‡Department of Paediatrics, S. Maria degli Angeli Hospital, Pordenone, the §Università Vita-Salute, San Raffaele Hospital, and the ||San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy.

Address correspondence and reprint requests to Marie O'Meara, MPharm, 20 Mercia Court, Highwood Close, London Se22 8NN, UK (e-mail: marieomeara2@yahoo.co.uk).

M.O. and M.C.P. contributed equally to the article.

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Portal hypertension and gastrointestinal bleeding (GIB) are major complications of chronic liver disease (1). Oesophagogastroduodenoscopy (OGD) and/or ileocolonoscopy can identify the source of bleeding in up to 95% of patients, allowing targeted treatment (2). When the source of bleeding is not obvious, presumably in the small bowel, the treatment options are limited. Consequences of persistent or recurrent bleeding in childhood include fatigue, poor school attendance/performance, and depression, secondary to chronic anaemia. Intractable bleeding necessitates multiple investigations, surgical interventions, and even consideration for liver transplantation (3). The associated mortality ranges from 2.5% to 20% (4). Treatment options for occult bleeding are limited, giving relevance to empirical pharmacological approaches (2,5–11).

Octreotide, the first somatostatin analogue introduced for clinical use, inhibits the release of growth hormone, glucagon, and insulin. Octreotide markedly reduces splanchnic blood flow and has a half-life 30- to 100-fold longer than somatostatin (12,13). It has a high affinity only to somatostatin receptors subtype 2 (sst2) and subtype 5 (14). sst2 is largely expressed in neuroendocrine and non-neuroendocrine cells of the human gastrointestinal (GI) tissue (15). Octreotide is licensed for symptomatic control and reduction of growth hormone and somatomedin C plasma levels in patients with acromegaly and for the relief of symptoms associated with functional gastroenteropancreatic tumours in adult patients (12). In children, octreotide has been used on an unlicensed basis for the treatment of hyperinsulinaemia, secretory diarrhoea, pancreatitis, chylothorax, and acute GIB either as a continuous infusion or 3 times daily subcutaneous injection (16–19). The long-acting octreotide (OCT-LAR) formulation of octreotide can be administered once per month with similar efficacy and safety profile to subcutaneous daily administration (20–22). Following a single intramuscular injection, OCT-LAR concentrations reach a plateau at day 14 and remain relatively constant for the following 3 to 4 weeks in adults (23). OCT-LAR therefore represents an attractive option for the long-term therapy of GIB. The use of OCT-LAR in children is limited owing to the lack of pharmacokinetic data, clinical experience, and several described adverse events, including nausea, abdominal cramps, diarrhoea, fat malabsorption, reduced glucose tolerance, and development of gallstones (20–24). Moreover, octreotide has the potential to interfere with endocrine mechanisms, an adverse effect particularly significant in the paediatric population. We report our experience with the use of OCT-LAR in treating severe GIB in children with portal hypertension secondary to chronic liver disease or portal vein thrombosis not responding to multiple conventional interventions, including variceal sclerotherapy or banding and intravenous or subcutaneous octreotide.

METHODS

The Paediatric Liver Service at King's College Hospital, London, UK, is a tertiary referral centre where some 500 new

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children with acute or chronic liver disorders are referred each year. From our database we have identified and reviewed retrospectively the clinical and laboratory data of all of the children in whom OCT-LAR, commercially available since 1998, has been used. Indications for OCT-LAR therapy were persistent GIB of unknown origin or resistant variceal bleeding previously treated with banding or sclerotherapy, with no or only partial response to intravenous octreotide.

Obscure GIB was defined as bleeding in the absence of an obvious cause and occult GIB as laboratory evidence of blood in the stools without macroscopic change in their appearance (10). The cause of bleeding was investigated using a combination of tests, as detailed in the Results section.

In the patients in whom oesophageal and/or gastric varices were identified, banding or sclerotherapy was performed. All of the patients with acute haemorrhage unresolved by sclerotherapy and banding were treated with a standard continuous intravenous octreotide infusion (50 μg/hour, irrespective of the weight), slowly weaned down 24 to 48 hours after bleeding stopped (11). If persistent rebleeding occurred after at least 4 sclerotherapy/banding sessions, necessitating frequent hospital admissions and numerous transfusions, the children were considered for treatment with OCT-LAR.

OCT-LAR was administered intramuscularly at a dose of 2.5 to 20 mg monthly. The initial dose was extrapolated from adult data for the treatment of acromegaly and neuroendocrine tumours, that is, 20 to 60 mg every 4 weeks (25,26). The paediatric dose was calculated as percentage of an average adult weight rounded to the closest 2.5 mg for ease of administration (not exceeding 20 mg). Blood transfusion was given when haemoglobin levels dropped <8 g/dL or when acute clinical bleeding occurred (1 transfusion unit = 250 mL blood). Response to treatment was assessed using the number of bleeding events, hospital admissions for acute bleeding episodes, and the number of blood units required during the year before and the year after initiation of therapy. Adverse effects during therapy were recorded retrospectively from the clinical notes.

Statistical Analysis

The primary endpoint was to determine the number of recurrent bleeding episodes in the 12 months after treatment with OCT-LAR compared with the previous 12 months. Secondary endpoints included the effect of OCT-LAR on the number of blood units transfused, on the number of hospital admissions, and on the length of hospital stay. Continuous variables are described by means of median values and ranges. Wilcoxon signed-rank test was used to compare the number of bleeding events, number of transfusions, number of hospital admissions, and number of days in hospital 1 year before and 1 year after OCT-LAR therapy. A P value <0.05 was considered significant.

RESULTS

Nine patients, 7 boys, who had received OCT-LAR from 2000 to 2009 were identified and studied. The median age at first bleeding was 21 months (range 1 month–14.5 years). Their diagnoses, treatment, and outcome are summarised in Table 1. All of the patients had a history of intermittent acute GIB (haematemesis, melaena, haematochezia), leading to severe anaemia requiring repeated blood transfusions. The underlying disease was biliary atresia in 7 (78%) children, 4 (57%) of whom had a successful (eg, leading to normalization of serum bilirubin) Kasai portoenterostomy, whereas the remaining 3 (43%) with persistent jaundice eventually required liver transplantation. Two of them developed portal hypertension as a consequence of portal vein thrombosis after liver transplant.

One child had Klippel-Trénaunay-Weber syndrome and thrombosis of the portal vein, whereas another had diffuse GI arteriovenous malformation complicated by arterialisation of the portal vein system and subsequently required partial hepatectomy.

All of the patients had portal hypertension characterised by recurrent bleeding and thrombocytopenia, with 7 patients reported to have splenomegaly. The remaining 2, patients 5 and 8, with no splenomegaly had polysplenia as part of their biliary atresia–splenic malformation syndrome. The universal presenting features were chronic anaemia (9 patients, 100%) and melaena (9 patients, 100%), whereas haematochezia was present in 7 (78%) and haematemesis in 6 (67%) children. There was no personal or family history of haemorrhagic disorders. Patient 4 was on long-term warfarin therapy because of the underlying Klippel-Trénaunay-Weber syndrome and chronic disseminated intravascular coagulopathy (27–29); patient 8 was temporarily treated with aspirin (8 mg/kg) because of a thrombus in the inferior vena cava and transiently high platelet count. After a 3-month treatment, aspirin therapy was discontinued because of anaemia, faecal occult blood, and thrombocytopenia, which continued following the withdrawal. Three patients (patients 6, 7, and 9) were on maintenance propranolol therapy ($0.5–1\ mg \cdot kg^{-1} \cdot day^{-1}$) both before and after treatment with OCT-LAR.

The tests performed to investigate the cause of bleeding included OGD in 9 (100%) patients, colonoscopy in 6 (67%), video capsule endoscopy in 4 (44%), angiography in 5 (56%), explorative laparotomy in 5 (56%), technetium-99m-labelled erythrocytes scintigraphy in 2 (22%), abdominal computerised tomography (CT) scan in 2 (22%), abdominal magnetic resonance imaging in 2 (22%), and barium meal and follow-through in 1 (11%).

A bleeding site was found in 5 cases (56%) (Table 1). Patient 1 had a vascular lesion, bleeding profusely, above the rectosigmoid junction. This was detected by colonoscopy and treated by electrocoagulation. Grade 3 oesophageal varices and grade 2 gastric varices were detected by OGD in patient 4 and the oesophageal varices were subsequently banded. Teleangiectasiae were also found by colonoscopy in the low caecum in this patient, but could not be treated. In patient 5 multiple bleeding lesions in the Roux loop with fresh blood and clots were detected during laparotomy, and in patient 6, multiple friable haemorrhagic polypoid lesions were found in the proximal part of duodenum, which could not be treated. In patient 8, grade 3 oesophageal varices were detected by OGD and treated with sclerotherapy. In all of the cases wherein the bleeding sites were treated, rebleeding occurred after the procedures with no reduction in the rate of episodes and blood transfusion requirements. In the remaining 4 children (44%) no active bleeding site was found, although nonbleeding oesophageal varices were seen in patient 2 during OGD and CT scans suggested the presence of Roux loop varices in patients 3, 7, and 9.

The median age at commencement of therapy with OCT LAR was 71 months (range 3–190 months). The median number of injections received was 13 (range 1–90). Three patients received only 1 dose. The median dose was 0.36 mg/kg (range 0.16–0.95 mg/kg). The patients have been studied for a median of 62 months (range 12–111 months) from initiation of the therapy (Table 1).

Three patients were excluded from statistical analysis: patient 1 because he had received subcutaneous octreotide immediately before starting OCT-LAR, patient 5 as he was only 3 months old at initiation of therapy, and patient 8 because his follow-up was shorter than 12 months. Patient 8 had received a single dose of OCT-LAR with no further bleeding, but underwent transplantation after 4 weeks. In the remaining 6 patients, the median number of injection was 12.5 (range 1–36), the median dose received for these

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TABLE 1. Patient characteristics, dosage of OCT-LAR, and outcome

Patient no. (sex)	Diagnosis	Age at first bleeding, mo	Source of bleeding	Age at first dose, mo	Dose, mg	Dose, mg/kg	No. doses	Follow-up posttherapy (no. mo)	Clinical details and outcome
1 (M)	BA successful Kasai	2.5	Throughout the gut	180	10	0.16	90	111	Subcutaneous octreotide for 11 mo before OCT-LAR Monthly OCT-LAR for 3 y, then 6-weekly to date No further GIB. Growth hormone deficiency, hypothyroidism.
2 (M)	BA, failed Kasai PVT post-LT	12	Oesophageal varices	29	5	0.36	1	80	LT at 10 mo of age. PVT 18 mo post-LT. Marked reduction of bleeding episodes after a single dose of OCT-LAR. PTLD then chronic rejection. Re LT at 7 years of age.
3 (M)	BA successful Kasai	21	Roux loop varices	35	5	0.35	15	69	No GIB during a 10-mo course of monthly OCT-LAR GIB 7 mo after stopping OCT-LAR, following a long-haul flight No further GIB with OCT-LAR administered 14 days before long-haul flights (>5 h)
4 (F)	KTW syndrome PVT	175	Grade 3 oesophageal varices, grade 2 gastric varices, telangiectasia in the lower caecum	190	20	0.29	15	62	Long-term warfarin treatment. PVT diagnosed at 14 y. Severe GIB ages 14–16 y Only 1 GIB episode during 15 doses of monthly OCT-LAR One episode 2 mo after cessation of therapy. No further GIB.
5 (M)	BA failed Kasai	1	Roux loop	3	2.5	0.46	1	14	Died of intracranial haemorrhage ages 17 mo, no further GIB. Post-mortem refused by family.
6 (M)	Liver+gut AVM portal vein arterialisation	60	Oesophageal, gastric, and duodenal varices	138	20	0.95	36	100	Microcephaly+developmental delay. Partial hepatectomy ages 3 y; banding of oesophageal varices ages 10 y. Propranolol 1 $mg \cdot kg^{-1} \cdot day^{-1}$. Monthly OCT-LAR from 11 to 17 y. No GIB after the first dose for 18 mo and then a marked reduction in the number and severity of episodes. From age 17, minor GIB episodes triggered by sharp food treated with OCT-LAR about 3 times per year.
7 (F)	BA, failed Kasai PVT post-LT	23	Roux loop varices	124	20	0.47	13	12	LT at 1 y of age. PVT 1 y post-LT. Unsuccessful portosystemic shunt at age 9 y. Patient was prescribed propranolol 0.5 $mg \cdot kg^{-1} \cdot day^{-1}$. Re-listed for LT and started on 4-weekly OCT-LAR

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TABLE 1. (continued)

Patient no. (sex)	Diagnosis	Age at first bleeding, mo	Source of bleeding	Age at first dose, mo	Dose, mg	Dose, mg/kg	No. doses	Follow-up posttherapy (no. mo)	Clinical details and outcome
8 (M)	BA failed Kasai	11	Oesophageal varices	12	2.5	0.33	1	31	Reduced GIB episodes for 6 mo, then GIB stopped. Removed from LT list. LT 4 wk after OCT-LAR administration with no further GIB before surgery
9 (M)	BA successful Kasai	23	Roux loop	71	10	0.4	13	12	OCT-LAR started after 6-mo severe melaena prompting listing for LT. Propranolol 0.5 mg · kg^{-1} · day^{-1}. No further GIB after the second dose and with monthly OCT-LAR injections to date. Removed from LT list.

AVM = arteriovenous malformation; BA = biliary atresia; GIB = gastrointestinal bleeding; KTW = Klippel-Trenaunay-Weber; LT = liver transplant; OCT-LAR = long-acting octreotide; PVT = portal vein thrombosis.

6 patients was 0.38 mg/kg (range 0.29–0.95 mg/kg), and the median follow-up was 65.5 months (range 5–100 months) from the initiation of treatment. In these 6 patients all parameters were analysed for the year before and the year after the initiation of therapy. The overall number of bleeding events decreased in all of the children after OCT-LAR therapy (Table 2) Two patients (patients 3 and 6) stopped bleeding immediately after the first dose of OCT-LAR; however, patient 3, whose OCT-LAR treatment was discontinued after 10 months, had a single bleed 7 months later. Patient 6, who had no reported bleeding episodes for 18 months after the first dose of OCT-LAR, started bleeding again, although the severity and frequency of the bleeding episodes were markedly reduced when compared with before receiving OCT-LAR. Patient 2 had a single dose of OCT-LAR with a conspicuous reduction in bleeding episodes and no further OCT-LAR was administered. The remaining 3 patients (patients 4, 7, and 9) stopped bleeding after 3, 7, and 2 injections (Table 1). The median number of blood transfusion units required per patient as well as the number of hospital admissions decreased in all of the children (Table 2). A reduction in the median length of hospital stay was also observed (Table 2). Five of 8 patients received monthly OCT-LAR injections for >6 months.

Excluding patient 1, who had already stopped bleeding while on subcutaneous octreotide before commencement of OCT-LAR, bleeding ceased in 7 of the remaining 8 patients: in 4 after the first injection of OCT-LAR (2 of whom received only 1 dose) and in 3 after a median of 3 doses (range 2–7 doses) (Table 1).

OCT-LAR was well tolerated in all of the patients. There were no reports of tachyphylactic reactions, gallstones, and liver or pancreatic disturbance (25). Apart from patient 1 (see below), no endocrine abnormalities were noted.

Patient 1 received subcutaneous octreotide (9.7 mg · kg^{-1} · day^{-1} in 3 divided doses) for 11 months before starting OCT-LA. As this was our first use of prophylactic subcutaneous octreotide, the dose was derived from the hyperinsulinaemia treatment (up to 40 μg · kg^{-1} · day^{-1}). Episodes of bleeding reduced from 219 during 12 months before subcutaneous octreotide to 14 in the first 7 months of subcutaneous therapy. Subsequently the bleeding stopped. OCT-LAR was started 11 months after subcutaneous octreotide therapy commenced and continues to the present day. Bleeding has never resumed, and after 3 years, the frequency of OCT-LAR was reduced to every 6 weeks. The family remains reluctant to stop therapy because of their concerns that GIB may re-occur. While being treated with subcutaneous octreotide, patient 1 developed diarrhoea and abdominal bloating, which resolved soon after the initiation of OCT-LAR. He was also diagnosed as having growth hormone deficiency during the first year of treatment with daily subcutaneous octreotide. At the age of 14 years, while being treated with subcutaneous octreotide, a growth hormone release test showed no growth hormone response, and his height velocity had

TABLE 2. Effects of OCT-LAR treatment on bleeding episodes in 6 patients (patients 2, 3, 4, 6, 7, and 9)

	Before therapy	After therapy	P
Observation period, mo	12	12	
No. GIB events	22 (12–110)	1.5 (0–27)	0.03
Hospital admissions for GIB	5.5 (2–7)	0.5 (0–2)	0.03
Length of hospital stay, days	47.5 (11–92)	2 (0–42)	0.03
Required blood units	5 (2–33)	0.5 (0–17)	0.03

Data are reported as median (range); Wilcoxon matched pairs test: $P < 0.05$ is statistically significant. GIB = gastrointestinal bleeding; OCT-LAR = long-acting octreotide.

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reduced markedly from 8.2 to 3.1 cm/y. Testicular volumes were appropriate for the pubertal growth spurt. Owing to the reduced height velocity, the decision was made to commence somatropin therapy for the remaining growth years. Fourteen months after starting subcutaneous octreotide, serum T4 level was at the lower limit of normal at 10.1 pmol/L (normal range 10–23 pmol/L), leading to the initiation of levothyroxine supplements (100 μg/day). The patient's growth and development has subsequently been normal (along the 50th percentile for height and 75th for weight throughout his adolescence).

At last follow-up, 7 patients were alive with no evidence of GIB: 3 on regular OCT-LAR treatment (patients 1, 7, and 9), 1 on intermittent (before long-haul flights) treatment (patients 3), and 2 off treatment (patient 2, retransplanted for chronic rejection 6 years after the first transplant and patient 4). Patient 6 has minor GIB episodes triggered by sharp food treated with OCT-LAR (approximately 3 injections per year). One patient had a liver transplant performed 4 weeks after starting OCT-LAR (patient 8). Two patients (patients 7 and 9), who had been listed for retransplant and transplant, respectively, as a result of severe persistent GIB, were removed from the transplant list because they stopped bleeding on OCT-LAR. One patient (patient 5) died of an intracranial haemorrhage of unknown cause 14 months after a single dose of OCT-LAR.

DISCUSSION

Our data provide novel evidence that OCT-LAR is effective, with no apparent serious adverse effects, in controlling severe GIB in children with advanced portal hypertension, in whom conventional endoscopic and medical treatments fail. Present international guidelines also include transjugular intrahepatic portosystemic shunting as a management option with considerable technical limitations in infants and small children (30), but in our centre, transjugular intrahepatic portosystemic shunting is considered only as a bridge to liver transplantation.

Continuous intravenous infusion of somatostatin analogues remains a standard therapy for acute GIB episodes in both adult and paediatric patients with portal hypertension (11,15–19). Subcutaneous octreotide therapy is effective in preventing recurrent bleeding episodes in adults (31–34), but the requirement for 3 times daily administration renders it impractical for long-term prophylaxis, particularly in children. Moreover, fluctuating octreotide serum levels have been reported with subcutaneous therapy (33). In adults, OCT-LAR administered on a monthly basis has similar efficacy to subcutaneous therapy, and results in sustained blood levels (21,32,33). These features of OCT-LAR prompted us to use it in children with refractory GI bleeding, despite the lack of paediatric studies for this indication.

In paediatrics, OCT-LAR has been used anecdotally for the treatment of Prader-Willi syndrome, but with no firm dose recommendations (24). The dose we used was extrapolated down for weight from that recommended for the treatment of acromegaly and neuroendocrine tumours in adults (median dose 0.36 mg · kg^{-1} · mo^{-1} [range 0.16–0.95]), corresponding to an adult dose of 23 mg/mo (range 10–60 mg/mo) (12,26). One child received a dose of 20 mg, 0.95 mg · kg^{-1} · mo^{-1} equivalent to 60 mg in an adult patient. This high dose, similar to that used in some patients with neuroendocrine tumours (26), was chosen because of the severity of his symptoms at the time of treatment initiation. The dose was not proportionally increased throughout his childhood because his symptoms remained well controlled; his present dose of 20 mg is equivalent to 0.6 mg/kg.

It is possible that OCT LAR doses lower than those used may have been equally effective, because in few adults in whom OCT-LAR has been used for GIB (35–38) both 20 and 10 mg monthly doses were reported to be effective (35–38). A randomised controlled study of 18 adult cirrhotic patients with portal hypertension showed a decrease in the hepatic venous pressure gradient (HVPG) in the treated arm, but not in the placebo arm using OCT-LAR at a dose of 20 mg monthly for a 3-month period (34). Although a similar proportion of treated and untreated patients (70% and 62% of octreotide- and placebo-treated patients, respectively) were taking β-blockers, it is possible that combined treatment with OCT-LAR and propranolol may have had an influence on the HVPG. In our study only 3 patients were taking propranolol at low doses during the year before and the year after starting OCT-LAR. Because we did not measure HVPG, we cannot comment on the possible cumulative effect of the β-blocker. In another study, on the assumption that the effect of octreotide on the GI tract—mediated by sst2A receptor subtypes—may be achieved by doses lower than those required to inhibit tumour growth, Scaglione et al (37) assessed the effectiveness of 10 mg/mo OCT-LAR in controlling chronic bleeding from GI angiodysplasias in 13 adult patients. During treatment a decrease in the number of transfusions as well as in the number and length of hospital admissions was noted; in 3 patients who did not respond to 10 mg/mo, the dose was increased to 20 mg/mo with no benefit.

The present study shows a significant reduction in the number of bleeding episodes in the year following the start of OCT-LAR treatment in all of the children, and, in addition, it shows complete cessation of bleeding in 7 of them. Most important, 2 children who were listed for liver transplantation because of the severity of their GIB were removed from the list and remain well with no further episodes of bleeding several months after starting OCT-LAR treatment.

As our study was not randomised and there was a great variation in the frequency and number of injections received by our patients, it is difficult to draw conclusions on how many doses are required to control bleeding. Among the 8 patients who stopped bleeding, 4 did so after the first injection of OCT-LAR, 2 of whom received only 1 dose (1 died and 1 was transplanted); 1 patient had already stopped bleeding on subcutaneous octreotide therapy before commencement of OCT-LAR; the remaining 3 stopped bleeding after a median of 3 (range 2–7) injections. For patient 2, who had a marked reduction of bleeding episodes after a single dose, the role of OCT-LAR remains speculative. We did not observe serious adverse effects immediately attributable to OCT-LAR treatment. Patient 5 died 11 months after a single dose of OCT-LAR, but, given the time interval between receiving OCT-LAR and death of intracranial haemorrhage, it is unlikely that the use of octreotide has contributed to his death. Patient 1 developed growth hormone deficiency and hypothyroidism during a prolonged period of treatment with a relatively high dose of subcutaneous octreotide (10 μg · kg^{-1} · day^{-1} compared with 0.16 μg · kg^{-1} · mo^{-1} while receiving OCT-LAR) before starting OCT-LAR. Because no further bleeding episodes occurred in the following 8 years of OCT-LAR therapy, it is possible that, had OCT-LAR been used initially, he would have stopped bleeding without developing adverse effects, as we observed in the remaining 8 patients.

In contrast with the lack of adverse effects attributable to OCT-LAR in our series, a recent article aiming at investigating the safety and efficacy of OCT-LAR in adult patients with Child-Pugh class A or B cirrhosis, no history of GI bleeding, and small oesophageal varices reports adverse events characterised by abdominal cramps, diarrhoea, and hypoglycaemia in 4 of 10 patients treated with a dose of 30 mg/mo, whereas no serious adverse effects were detected in patients treated with 10 mg/mo (39). In the same multicentre study, no haemodynamic benefit with

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either OCT-LAR dose was demonstrated by measuring HVPG, but the authors emphasise that the quality of the HVPG tracings across centres was variable making interpretation difficult. Although both the endpoints and the patient characteristics in that study are different from those in our report, it is intriguing that no abdominal adverse effects or hypoglycaemia were reported in any of our children. It is important to note, however, that only 3 of our 9 patients received an equivalent OCT-LAR dose ≥30 mg/mo if extrapolated. We suggest, therefore, that caution should be exercised before using doses at the higher end of the spectrum.

Owing to its small size and its retrospective nature, this study is unable to recommend an optimal length of OCT-LAR treatment vis-à-vis the recurrence of bleeding and the risk of adverse effects, but an empirical approach with monitoring of the thyroid function and growth velocity appears to be prudent.

In conclusion, this pilot experience indicates that OCT-LAR may be effective in controlling severe recurrent GIB not amenable to conventional endoscopic treatment in children with portal hypertension caused by chronic liver disease and GI vascular malformations. This therapy is less invasive than daily subcutaneous octreotide injections, reducing compliance issues. Adverse effects are uncommon but regular endocrine assessment, particularly in adolescent patients, is recommended. Pharmacokinetic studies and prospective randomised controlled trials are necessary to establish the optimum dose and length of treatment of OCT-LAR and confirm its efficacy and long-term safety in treating severe GIB in children.

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Abnormalities of acid–base balance and predisposition to metabolic acidosis in Metachromatic Leukodystrophy patients

L. Lorioli [a,b,c,d], M.P. Cicalese [a,b], P. Silvani [e], A. Assanelli [b,c,g], I. Salvo [i], A. Mandelli [i], F. Fumagalli [a,f], R. Fiori [e], F. Ciceri [g], A. Aiuti [a,b,c,d], M. Sessa [a,f], M.G. Roncarolo [a,b,c,d], C. Lanzani [h], A. Biffi [a,b,c,*]

[a] San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Hospital, Milano, Italy
[b] Pediatric Immunohematology Unit, San Raffaele Hospital, Milano, Italy
[c] Stem Cell Transplantation Program, San Raffaele Hospital, Milano, Italy
[d] Vita-Salute San Raffaele University, Milano, Italy
[e] Departement of Anesthesia and Critical Care, San Raffaele Hospital, Milano, Italy
[f] Neurology Department, Division of Neuroscience, San Raffaele Hospital, Milano, Italy
[g] Bone marrow Transplantation Unit, San Raffaele Hospital, Milano, Italy
[h] Nephrology Department, San Raffaele Hospital, Milano, Italy
[i] Pediatric Anesthesia and Intensive Care, Buzzi Children Hospital, Milano, Italy

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ABSTRACT

Metachromatic Leukodystrophy (MLD; MIM# 250100) is a rare inherited lysosomal storage disorder caused by the deficiency of Arylsulfatase A (ARSA). The enzymatic defect results in the accumulation of the ARSA substrate that is particularly relevant in myelin forming cells and leads to progressive dysmyelination and dysfunction of the central and peripheral nervous system. Sulfatide accumulation has also been reported in various visceral organs, although little is known about the potential clinical consequences of such accumulation. Different forms of MLD-associated gallbladder disease have been described, and there is one reported case of an MLD patient presenting with functional consequences of sulfatide accumulation in the kidney.

Here we describe a wide cohort of MLD patients in whom a tendency to sub-clinical metabolic acidosis was observed. Furthermore in some of them we report episodes of metabolic acidosis of different grades of severity developed in acute clinical conditions of various origin. Importantly, we finally show how a careful acid–base balance monitoring and prompt correction of imbalances might prevent severe consequences of acidosis.

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1. Introduction

Metachromatic Leukodystrophy (MLD; MIM# 250100) is a rare inherited lysosomal storage disorder caused by the deficiency of Arylsulfatase A (ARSA). The enzymatic defect results in the accumulation of the ARSA substrate galactosylceramide I3-sulfate, also known as sulfatide, in the lysosomes of all cell types. Substrate storage is particularly relevant in myelin forming cells, both oligodendrocytes and Schwann cells, leading to progressive dysmyelination and dysfunction of the central (CNS) and peripheral (PNS) nervous system, respectively [1]. However, sulfatide accumulation has also been reported in various visceral organs (liver, gallbladder, kidney, pancreas, lymph nodes, adrenal glands, and ovaries) of post-mortem MLD patients [2,3], even though little is known on the potential clinical consequences of such accumulation. Some clinical reports describe MLD-associated gallbladder disease in different forms such as thickening of the gallbladder wall, gallstones, and polyposis [4–8], however, in only one case has an MLD patient been reported as presenting a functional consequence (moderate and persistent metabolic acidosis due to proximal tubular dysfunction) of sulfatide accumulation in the kidney [9].

Here we describe MLD patients who display sub-clinical metabolic acidosis at baseline and eventually develop overt metabolic acidosis of different grades of severity in acute clinical conditions, and show how a careful acid–base balance monitoring and correction of imbalances might prevent severe consequences of acidosis.

2. Patients and methods

The cohort of subjects here described is composed of 24 patients affected by late infantile (LI) (13/24) or early juvenile (EJ) (11/24) MLD, followed at our Institute within a natural history study [10] and more recently in the context of a phase I/II clinical trial of autologous haematopoietic stem cell gene therapy [11]; both clinical protocols

Abbreviations: ARSA, Arylsulfatase A; CNS, central nervous system; EJ, early juvenile; LI, late infantile; MLD, Metachromatic Leukodystrophy; PNS, peripheral nervous system; Pts, patients.

* Corresponding author at: HSR-TIGET, San Raffaele Scientific Institute, Via Olgettina 58, 20132, Milan, Italy.
E-mail address: biffi.alessandra@hsr.it (A. Biffi).

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were approved by the medical Ethic Committee of the San Raffaele Hospital in Milan. Fifteen patients are of Caucasian ethnicity (4 Italians, 4 Americans, 2 British, 2 Swedish, 1 Norwegian, 1 Polish, 1 Czech), 6 are Arab, 2 are of Hispanic origin (Brazilian), and one patient is of Turkish origin. The age range at observation is between 8 months and 18 years old.

All of the patients were retrospectively evaluated for acid–base balance in different phases of their disease, either very advanced in the case of the natural history study or before/in the early phases after symptom onset in the case of their enrolment in the gene therapy clinical trial. Furthermore, the patients enrolled in the gene therapy clinical trial were prospectively monitored for acid–base balance at +7 days and +3 months after gene therapy. Analyses performed on blood samples include acid–base balance parameters (pH, base excess, bicarbonates), serum electrolytes (sodium, chloride, potassium), creatinine, creatinine clearance (calculated with the Schwartz formula, mL/min per 1.73 m^2), and lactate; on urine samples pH, sodium, chloride, potassium and chemistry were evaluated. For patient #1 renin, aldosterone, dosage of essential amino acids in blood and urine, thyroid function and dosage of vitamin B1 were tested in the acute phase. For all these tests reference values of our internal laboratory (LaboRaf, San Raffaele Hospital, Milan, Italy) were used.

3. Results

Over the past 10 years, we have followed patients affected by MLD within a natural history study [10] and more recently in the context of a phase I/II clinical trial of autologous haematopoietic stem cell gene therapy [11]. In some of these patients, as part of their routine clinical management, acid–base balance was monitored by venous (pH, base excess, bicarbonate levels) and urinary (pH) assessments. In the context of the same routine management, serum electrolytes (Na, K, Mg, Ca, Cl) and creatinine clearance were repeatedly assessed in some of the patients. A total of 24 patients affected by late infantile (LI) or early juvenile (EJ) MLD were tested in different phases of their disease, either very advanced in the case of the natural history study or before/in the early phases after symptom onset in the case of their enrolment in the gene therapy clinical trial. For some of the patients repeated measurements were also collected during their follow-up, i.e. when they experienced acute clinical conditions.

3.1. Acid–base balance and acidosis in MLD patients in acute conditions

During their clinical course and follow-up, seven of the 24 patients (29%) developed a clinically-relevant metabolic acidosis, requiring supplementation of bicarbonates for either clinical management of acute clinical signs and symptoms, or in a preventive fashion based on laboratory venous acid–base findings. In all of the cases acidosis was documented in close association to an acute and stressful clinical event (Table 1). Also in the case of the patients enrolled and treated in the gene therapy clinical trial (Pts 1–6), acidosis occurred in occasion of acute events, such as main surgery or sepsis, either before or shortly after the administration of the chemotherapy regimen and of the autologous gene-modified cells envisaged by the gene therapy treatment (Table 1). In particular, treatment consisted of transplantation of autologous gene-modified haematopoietic stem cells after the administration of a myeloablative, dose-adjusted, intravenous (iv) busulfan chemotherapy. During the neutropenic phase, transplanted patients received prophylaxis for bacterial (Amoxocilin-clavulanic acid 50 mg/kg/day), viral (Acydovir 15 mg/kg/day), *Pneumocystis jirovecii* (Pentamidin aerosol 9 mg/kg every 3 weeks) and fungal (liposomal B Amphotericin 1 mg/kg/day iv) infections.

Patient # 1 experienced two events of acute acidosis during severe episodes of febrile neutropenia and sepsis (day +11 and +37 post-transplant). High lactate levels in association with acidosis (pH 6.9%; bicarbonate 6 mmol/l, base-excess −28.8 mmol/L, pCO2 15.8 mm Hg, Lactate 14 mmol/L at first episode; pH 7.24%, bicarbonates 12.8 mmol/L, base-excess −13.3 mmol/L, pCO2 30.2 mm Hg and lactate 12.53 mmol/L at second episode) were detected in venous blood (Fig. 1). Urinary testing showed loss of glucose in urine in the presence of a normal serum glucose level. The first episode promptly resolved upon administration of bicarbonates and dedicated support measures (cristalloids, blood products, diuretics). Thereafter, she maintained a good acid–base balance without the need for bicarbonate supplementation until the second episode of sepsis and acidosis when iv bicarbonates were again employed for acute episode management. Upon resolution of the clinical event, bicarbonate supplementation was progressively decreased and completely stopped 18 months after transplant. Alkalinisation of urine (up to pH values of 8.5%) upon bicarbonate supplementation was documented. Of note, at a retrospective evaluation of patient's serum and urinary acid–base balance (2 months before treatment), we noticed pre-existing urinary borderline pH (equal to 7%) and elevated sodium renal excretion, suggesting that urinary loss of bicarbonates could have been already present in baseline conditions and that possibly it could not have been compensated for a stress situation. These findings associated with elevated sodium excretion, and in the absence of any other organic abnormality, were considered suggestive of a type II tubulopathy affecting the proximal tubule with decreased resorption of bicarbonates. To investigate whether another underlying cause of lactic acidosis was present and to understand the tubular defect in more detail, measurement of the levels of essential amino acids in blood and urine was performed, showing normal levels of amino acid in blood and increased level of urinary amino acids, compatible with type II tubulopathy. All the other kidney laboratory parameters including creatinine clearance were within normal limits. Renin, aldosterone, potassium, calcium, proteins and the calcium/creatinine ratio in urine were within normal limits. Thyroid function and level of vitamin B1 were normal. We thus confirmed our diagnostic hypothesis of a tubular defect causing urinary bicarbonate loss, likely associated with kidney sulfatide accumulation.

Based on this clinical experience, we prospectively started a blood and urinary acid–base balance monitoring in all subsequent patients, thus allowing prompt identification and correction of acidosis when needed, as what happened in the cases described below.

Patients from #2 to #6 showed a tendency to alkaline urinary pH (data not shown) and borderline low serum pH, bicarbonate and base

Table 1
Acute patients' characteristics. +/− indicates timing of the acute acidosis episode as respect to gene therapy (GT), where "Ch." stands for the day of initiation of chemotherapy and "Tx" indicates the day of transplantation of the gene corrected cells; d = days; hr = hours; mo = months. BM = bone marrow.

Patients	MLD variant	1st finding of acidosis		
		Age	Concomitant event	Before/after GT (or no GT)
1	Symptomatic EJ	5years	Sepsis	After Tx (+11 and +37 d)
2	Pre-symptomatic LI	15 ms	Febrile neutropenia	After (+9 d)
3	Pre-symptomatic LI	23 ms	Febrile neutropenia	After (+9 d)
4	Symptomatic EJ	11year	Main surgery (BM explant)	Before Ch. (−6 hr) and Tx (−4 d)
5	Pre-symptomatic LI	8 ms	Sepsis	Before Ch. and Tx (−2 mo)
6	Symptomatic EJ	7years	Main surgery (BM explant)	Before Ch. (−6 hr) and Tx (−4 d)
7	Late symptomatic LI	3.2 years	Status epilepticus	No GT

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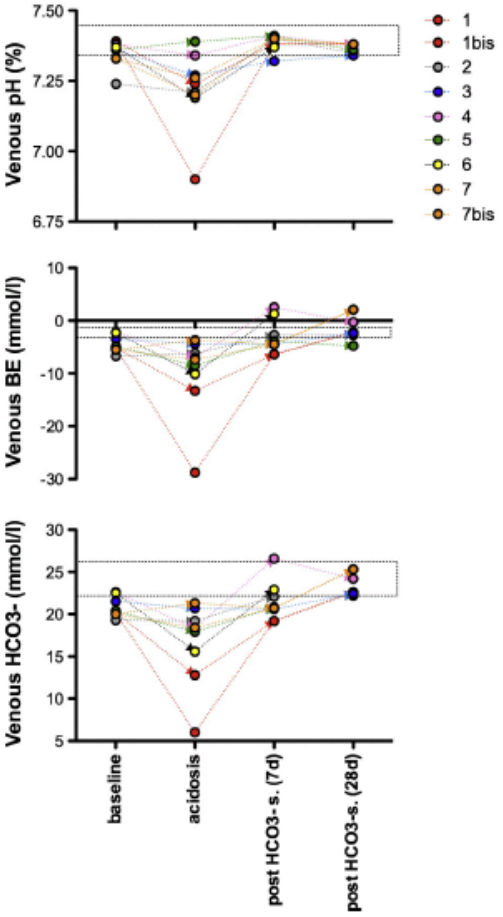

Fig. 1. Acid–base balance in patients who developed overt metabolic acidosis in acute conditions. Venous pH, base excess (BE) and bicarbonate (HCO_3^-) values of the 7 patients who developed overt metabolic acidosis in acute clinical conditions are reported at baseline evaluation, at the time of starting bicarbonates supplementation (acidosis), and then at +7 and +28 days after the beginning of bicarbonate supplementation (post-HCO_3^- s. (7 or 28 days)). Bicarbonates administration allowed restoration not only of bicarbonates levels but also of serum pH and base excess to (close to) normal values in all the treated cases. Reference range for each parameter is reported as dotted rectangles.

excess values before the autologous transplantation of gene corrected haematopoietic stem cells (Fig. 1).

In the case of Patients # 2 and 3 acidosis was manifested in acute events occurring after gene therapy administration. Starting on day +9 post-transplant they experienced oral mucositis (grade III in Patient #2, grade I in Patient #3), and subsequently developed febrile neutropenia. Importantly, during this acute phase all three patients showed low plasma pH and/or bicarbonate and/or base excess values, with consequent need for bicarbonate supplementation (Fig. 1); we also observed a tendency to high urine pH values (≥7.5%) associated with bicarbonate supplementation. After one month bicarbonate supplementation was progressively converted to oral administration and then stopped when an acid–base balance within normal limits was achieved. Urinary amino acids were not tested because of poor compliance of the patients. All renal laboratory parameters tested, as for Patient #1, including lactate levels, were within normal limits.

Patients #4, 5 and 6 developed acidosis (low plasma bicarbonate values and/or low pH and/or below normal base excess value) in occasion of acute clinical events occurring in the preparatory phase of gene therapy, before starting the treatment: in occasion of main surgery for bone marrow collection (Patients #4 and 6) and of a septic episode (central line infection) occurring 2 months before initiating the gene therapy procedure (Patient #5). Also in their case bicarbonates supply was introduced with good response (Fig. 1).

Patient # 7 is a 3.2 yeas-old LI MLD child in an advanced stage of the disease. Tendency to mild acidosis (pH 7.33%, base excess −5.5 mmol/L), borderline bicarbonate level (20 mmol/L), and alkaline urinary pH came to light at a baseline screening. He experienced several episodes of upper airway infection, but he had a quite good compensation of the acid–base balance. However, in preparation for a surgical intervention for percutaneous gastrostomy positioning, we re-tested the acid–base balance and we found a manifested state of acidosis (pH 7.20%; base excess −7.4 mmol/L; bicarbonate 18 mmol/L). We thus started oral bicarbonate supplementation with prompt correction of the acidosis. A few days later he experienced status epilepticus, in the context of which metabolic acidosis with urinary bicarbonate loss exacerbated despite ongoing oral supplementation (pH 7.26%; base excess −3.7 mmol/L; bicarbonate 21.3 mmol/L, urinary pH 8.5%). We thus increased bicarbonate supplementation (up to a maximum of 3 mEq/kg/day, based on the daily acid–base monitoring), with correction of acidosis. We cannot exclude the presence of unrecognized seizure activity at the time of first detection of acidosis that could have later evolved into a clinical status epilepticus.

Of note, we could calculate anion gap during the episodes of metabolic acidosis in 5 out of 7 patients. In 3 out of 5 patients (Patient #1, Patient #3, Patient #6) we observed elevated anion gap acidosis that, as expected, normalized with the resolution of the acute event and upon bicarbonate supplementation.

3.2. Acid–base balance and evidence of acidosis in MLD patients during baseline conditions

Based on the findings in these acute/sub-acute patients, we then retrospectively analysed the acid–base data collected over the years in our patient cohort under baseline clinical conditions, namely when the patients were not experiencing acute infections or illnesses (apart from their underlying MLD disease). Data from 17 patients were available and multiple data points were retrieved for some of the subjects. These subjects were in different stages of MLD since some were followed in the context of the natural history study and were thus generally in advanced disease stage, while others were enrolled in the gene therapy clinical trial and were thus analysed before beginning of the treatment in an early stage of the disease (Fig. 2, baseline values). The tested patient population showed normal creatinine and creatinine clearance (calculated with Schwartz formula), and on average barely normal venous pH (7.35 ± 0.05%), base excess (−2.48 ± 2.05 mmol/L) and bicarbonate (21.8 ± 1.8 mmol/L) values. Interestingly, however, when individual subjects were considered a great fraction of them showed values below the normal range for each of the tested parameters, independently from their disease variant and stage at testing (Fig. 2). In particular, in 54% of the cases recorded bicarbonate levels were below the reference range (from 22 to 26 mmol/L) and 47% of the collected base excess values landed below the normal range (from −2 to −3 mmol/L). In only 22% of the cases pH fell below the reference range (from 7.33 to 7.43%). None of the patients showing low venous base excess or bicarbonate or pH levels in baseline conditions manifested clinical or other clinical chemistry signs of acidosis, or acute clinical conditions that could have contributed to the development of these abnormalities. Of note, urine pH was on average 6.4 ± 0.9%, with 58% of pH values above 6 (data not shown).

Venous acid–base balance was then analysed short-term after gene therapy (at 7 days and 3 months) in those patients within this cohort who underwent gene therapy (9 subjects) in order to assess whether

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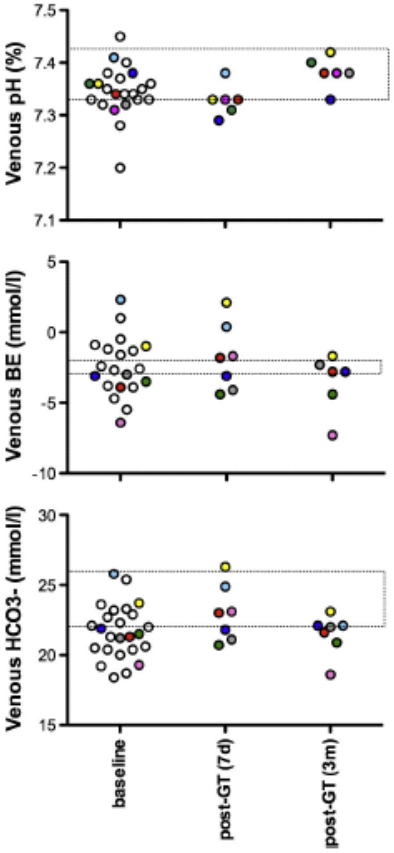

Fig. 2. Acid–base balance in MLD patients during baseline conditions. Venous pH, base excess (BE) and bicarbonate ($HCO3^-$) values of the cohort of MLD patients: coloured dots = patients enrolled in the gene therapy clinical trial, white dots = patients enrolled in the natural history study at baseline conditions, free of any acute illness. For patients who underwent gene therapy the same parameters are reported at short- (+7 days) and medium-term (+3 months) follow-up intervals after the transplant. No significant variation in acid–base balance parameters after transplantation as compared to baseline evaluations could be detected. Reference range for each parameter is reported as dotted rectangles.

the procedure per se or any of the drugs employed for pre-transplant conditioning, for prevention of post-transplant infections and for post-transplant management could have affected the tested parameters and contributed to the severe acidosis observed post-transplant in Patients # 1, 2 and 3. Samples were evaluated in gene therapy-treated patients not showing acute illnesses and not receiving alkali supplementation. No significant reductions in venous pH, base excess and bicarbonate levels were observed either at very short term (7 days) or at 3 months post-transplant (Fig. 2).

4. Discussion and conclusion

The ARSA enzyme is involved in the catabolism of membrane sulfatides and its deficiency causes accumulation of undegraded substrate in the lysosomes in all cell types. Sulfatide is a major sphingolipid of myelin and its accumulation in the CNS and PNS is responsible for the severe neurological manifestations of MLD; on the contrary little is known about the clinical consequences of sulfatide accumulation on visceral organs [4,5]. Sulfatide degradation normally occurs in the kidney and large quantities of sulfatides can be detected in the urinary sediment of affected individuals. Indeed, measurement of sulfatide excretion in urine is part of the recommended diagnostic workup for MLD [5,12], together with ARSA gene sequencing and ARSA activity determination. Furthermore, at historical histo-pathological kidney evaluations of post-mortem MLD patients metachromatic granules were described in the cytoplasm of convoluted tubules, loops of Henle and collecting tubules, with a patchy distribution [2,3].

The knowledge on non-nervous system manifestations in MLD is limited, probably because of the short lifespan of early onset patients and because visceral manifestations may receive less attention in the context of the devastating neurological symptoms. Gallbladder involvement in MLD affected children, and, mainly, in adults has been widely documented [5,12]. In contrast, renal manifestations of MLD have not been well described, and only one 2 year-old patient has been reported, presenting with a moderate and persistent metabolic acidosis due to proximal tubular dysfunction related to sulfatide accumulation [9]; two additional cases have been described where renal sulfatide deposits were associated with a chronic nephritis that was not further specified [13,14]. No description of renal function in a large series of MLD patients has been reported until now.

Here we report the occurrence of acute metabolic acidosis under stressful clinical conditions in a series of 7 MLD patients. No specific association of these acute acidosis episodes with a specific variant of the disease (late infantile versus early juvenile disease), disease stage (pre-symptomatic versus early/advanced symptomatic) and/or cause of the acute stress preceding the episode (surgical, septic, neurological/seizure-related) has been identified. Interestingly, the observation of these few acute episodes highlighted a clinically relevant renal defect associated to the disease. Indeed, at a subsequent retrospective evaluation of the entire cohort of MLD patients followed at our Institution (participating in the natural history and gene therapy studies), analysed in baseline conditions, when no acute illness other than their primary disease was present, a tendency to sub-clinical metabolic acidosis was observed. The evidence that i) a large fraction of the baseline values of our cohort falls below the reference range for normal acid–base balance, ii) a mild alkalinisation of the urine is present in a large proportion of our cases, and iii) no obvious respiratory or metabolic co-factors involved in the determination of the observed mild acidosis could be identified, together with the results of the characterization of renal function performed in a few of the patients may indicate a defective tubular reuptake of bicarbonate in MLD patients.

Importantly, our series suggests that metabolic stress such as the one associated to febrile or septic episodes, or seizure activity, or other major events such as surgery could exacerbate the latent kidney tubule dysfunction present in baseline conditions, leading to an acute increase in the loss of bicarbonate and to a rapid worsening of metabolic acidosis and of its clinical consequences, if prompt correction is not established. Bicarbonate administration allowed restoration not only of bicarbonates levels but also of serum pH and base excess to approximately normal values in all of the treated cases, and contributed to clinical recovery in the cases were acidosis was clinically manifested. Importantly, the impaired functional state was reversible when the stressful event ceased, as what happened in our patients who gain independence from bicarbonate supplementation after resolution of the acute clinical condition.

The analysis of the patients treated by autologous transplantation of gene corrected cells who had not developed acidosis and who were analysed at resting conditions short- and medium-term after the transplant is also of great relevance. Indeed, the lack of any significant variation in acid–base balance parameters after gene therapy would indicate that the treatment per se as well as the drugs employed in routine gene therapy management do not play a critical role in the exacerbation of tubular reabsorption of bicarbonates, that is rather caused by the stressful events occurring either before or after gene therapy, or in Patient #7 independently from such procedure.

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Based on these findings and laboratory results, a proximal tubular defect and consequent acidosis associated with urinary bicarbonate loss, likely due to disease-related kidney accumulation of sulfatides, was hypothesised. The relatively high urine pH values registered before bicarbonate supplementation may still be compatible with this hypothesis of a proximal tubular dysfunction in the paediatric age, when a good skeletal compensation could contribute. This diagnosis is also consistent with the exacerbation of the clinical/laboratory findings upon acute stress (i.e. during septic state or status epilepticus).

The diagnosis of tubular dysfunction in MLD patients does not require an invasive diagnostic procedure, such as kidney biopsy, but necessitates prompt recognition by accurate monitoring of acid–base balance, and subsequent targeted treatment. Knowledge of this manifestation of the disease is of great relevance in view of new therapeutic approaches currently being tested for this disease, such as haematopoietic stem cell transplantation [15–18], gene therapy [11] and enzyme replacement strategies (ClinicalTrials.gov Identifier: NCT01510028). Indeed, along with the application of new and intense therapeutic opportunities to these patients, potential prolongation of their life expectancy and amelioration of their prognosis may render them more susceptible to episodes of acute acidosis due to the treatment-related and/or treatment-independent exposure to stressful situations. Finally, as relatively little attention has been paid to biochemical abnormalities of patients with a such devastating neurological picture, we recommend a careful evaluation of this aspect in all MLD subjects, especially in those in an advanced stage of the disease or undergoing surgical procedures or other intensive treatments. A careful monitoring of acid–base status is thus crucial for these patients, particularly during acute clinical illnesses or when undergoing invasive therapeutic treatments, in order to prevent acute worsening of clinical conditions and immediately provide an adequate support in all the relevant different settings and stages of the disease. Our experience could also be relevant in the context of other lysosomal storage disorders in which kidney function could be similarly affected.

Conflict of interest

None of the authors declares any conflict of interest.

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BRIEF REPORT

TITLE Fatal CMV pneumonia due to primary infection in a CMV negative recipient (R) /donor (D) pair after hematopoietic stem cells transplantation.

AUTHORS

Cicalese MP, MD[1,2], Fossati M., MD[1,3], Assanelli A., MD[1,4], Biffi A., MD[1,2], Marktel S., MD[4], Fiori R., MD[5], Fumagalli L., MD[6], Aiuti A, MD, PhD[1,2,3], Ciceri F, MD[2,4].

AFFILIATIONS: [1]Pediatric Immunohematology and Bone Marrow Transplant Unit, San Raffaele Scientific Institute, Milan, Italy; [2]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy; [3]Vita-Salute San Raffaele University, Milan, Italy; [4]Haematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan, Italy; [5]Department of Anestesiology, San Raffaele Scientific Institute, Milan, Italy; [6]Department of Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy.

KEYWORDS: CMV, children, hematopoietic stem cell transplantation

RUNNING HEAD: Primary CMV infection after HSCT

CORRESPONDING AUTHOR: ciceri.fabio@hsr.it

ABSTRACT

In the era of surveillance and anti-infective prophylaxis in the hematopoietic stem cell transplantation setting, the onset of human cytomegalovirus (CMV) primary infection in a negative recipient (R) /donor (D) pair after transplantation has become extremely rare. We report a case of CMV primary infection in a CMV negative couple (R/D) followed by a fatal CMV pneumonia and discuss some possible strategies to decrease the risk of primary CMV infection and to improve its management in bone marrow transplantation centres.

MAIN TEXT

INTRODUCTION

Human cytomegalovirus (CMV) infection remains one of the major life-threatening complication following hematopoietic stem cell transplantation (HSCT) (1). Frequent clinical manifestations of CMV disease are interstitial pneumonia, gastrointestinal disease (GI) and hepatitis, retinitis, encephalitis, and marrow suppression (2). Pneumonia is the most frequent cause of death due to CMV disease in patients undergoing immunosuppressive therapy and its management still remains very challenging (2).

Pre-transplant CMV serostatus is actually the most important determinant of CMV reactivation and CMV disease after HSCT. Even in the era of surveillance and preemptive therapy, CMV reactivation approaches 70% of recipients who are CMV sieropositive (R+) and/or receive a transplant from a CMV-seropositive donor (D+) and 25% of patients develops CMV disease with a high rate of morbidity and mortality (3, 4).
On the contrary, primary infection in recipients who are CMV seronegative (R-) and receive a transplant from a CMV seronegative donor (D-) is an extremely unlucky and rare event (4).
The available strategies to prevent a primary infection in a CMV seronegative recipient are to choose possibly a CMV seronegative donor and to transfuse, when possible, with CMV seronegative or leukocyte-depleted blood products (5, 6). Using these strategies, the overall risk of primary CMV infection in the recipient drops to 1-4% (6-8), but these cases have been rarely described and there is lack of detailed information in literature about them.

In this paper, we report a case of CMV primary infection in a CMV negative (R/D) pair followed by a fatal CMV pneumonia and we provide a review of the currently used and most recent approach to prevent and treat CMV primary infection in bone marrow transplantation setting. Moreover, we discuss some possible strategies to decrease the risk of primary CMV

infection in R/D negative pairs and to improve its management in bone marrow transplantation centres.

CASE REPORT

The patient was a 11 years old child with high risk B Common Acute Lymphoblastic Leukemia (ALL) referred to our Institution to receive a haploidentical HSCT. She was diagnosed almost 2 years before and treated in line with the protocol LAL 2000 of the Italian Association of Pediatric Ematology and Oncology, AIEOP (9). She experienced an early relapse during treatment. She then received a second line regimen using Fludarabine, high dose cytarabine (HD ARA-C), granulocyte stimulating factor (GCSF) and methotrexate (10), but the disease relapsed again. Due to the chemo-refractoriness, the ineffective re-induction attempts and the unavailability of suitable matched sibling and familiar donors (MSD and MFD) and HLA-matched unrelated donors (MUDs), she was offered a haploidentical T-depleted SCT from her father.

One month before her arrival to our Institution, the patient developed a Staphylococcal pneumonia documented by chest CT scan and positive blood cultures, treated with teicoplanin (10 mg/kg/d) and piperaciline-tazobactam (300 mg/kg/d) and achieving a chest CT scan negativity. Before HSCT, serological viral tests for CMV, EBV, HBV, HCV and HIV were performed and both the recipient and the donor resulted CMV IgG and IgM negative.

The conditioning regimen consisted of thiotepa 10 mg/kg on day -7; treosulphan 14 g/m^2 on days -6, -5, -4; fludarabine 30 mg/m^2 on days -6, -5, -4, -3, -2; rituximab 200 mg/m^2 on day -1; rabbit antithymocyte globulin Fresenius® (ATG) 10 mg/kg on days -4, -3, -2. Graft-versus-host disease (GVHD) prophylaxis consisted of rapamycine (starting dose 2 mg/kg from day -7) and mycophenolate mofetil (15 mg/kg/day from day 0). Anti-infective prophylaxis consisted of ceftriaxone 50 mg/kg; cotrimoxazole at a trimetoprim dose of 5 mg/kg 2 days/week from

day -9 to -2, followed by nebulized pentamidine 250 mg every 3 weeks; acyclovir 15 mg/Kg/day and voriconazole 200 mg/day.

Considering the host/donor negative CMV status, CMV surveillance consisted of polymerase chain reaction CMV detection once every 2 weeks and the use of irradiated, filtered and leukocyte-depleted blood products.

The patient was hosted in a single room, with a private toilet, and mother and father were admitted in the room alternatively, as recommended by Centers for Disease Control and World Health Organization (11). The graft contained 7.04x10e6 CD34+/kg cells. Neutrophil engraftment with an absolute neutrophil count >0.5x10e9/L was documented 22 days after transplant. Thrombocyte engraftment with platelets >20x10e9/L never occurred. On day 7 after SCT, she developed high fever and an elevation of CRP (23,4 mg/L, normal values < 6 mg/L). Empirical large spectrum antibiotic therapy with ceftazidime (100 mg/kg/d) and teicoplanine (10 mg/kg/d) was started. No positive blood cultures were found. On day + 10 from SCT, a generalized rash characterized by pinky plaques was detected associated with an increase in liver enzymes levels (AST 113 U/L, ALT 121 U/L). PCR test for CMV resulted in more than 10 x 10^6 copies/ml indicating a primary CMV infection. Foscarnet (180 mg/kg/day) and anti-CMV hyperimmune gammaglobulin 1400 U/week (Cytotect®) were started. A chest CT scan was performed and resulted negative **(Figure 1A)**. CMV PCR was confirmed positive (> 10 x 10^6 copies/ml) in 2 further determinations and parents were tested for CMV by ELISA and PCR, but resulted negative.

Persistent fever and septical status required several and consecutive changes of the antibiotic therapy - from ceftazidime (100 mg/kg/day) to piperacilline-tazobactam (300 mg/kg/day) and then to meropenem (60 mg/kg/day); shift from teicoplanin (10 mg/kg/day) to linezolid (15 mg/kg/day) – but no other cultural isolation was detected. On day + 16 from SCT, the patient started to develop an insistent cough and an increased respiratory rate; fine crackles were present on lung examination and a high level of CRP was detected (225 mg/L). The patient started to require oxygen therapy and the general conditions worsened. Another CT scan was performed (day + 17) and revealed a bilateral pleural effusion and

bilateral thickening due to disventilation. On day + 18, Foscarnet was discontinued in order to decrease renal toxicity and fluid retention, and Ganciclovir (GCV) (10 mg/kg/day) was started. CMV was tested for chemosensitivity and no drug resistance was found. The clinical situation continued to worsen in spite of good engraftment and the patient was started on non-invasive positive pressure ventilation (CPAP – PEEP 7.5 FiO2 40%). Another chest CT scan (day + 20 from SCT) was performed and revealed an important bilateral pleural effusion until the apexes, bilateral patchy ground glass opacity and interstitial oedema. The pleural effusion was drained bilaterally determining an improvement of clinical condition even if a progressive air space consolidation was documented on a further chest CT scan, performed on day + 25 from SCT **(Figure 1B)**.

On day + 22 from SCT, myeloid chimerism on peripheral blood and bone marrow aspirate resulted full donor. PCR test for CMV revealed a decrease from 4 x 10^6 copies/ml (day + 24 from SCT) to 0.3 x 10^6 copies/ml (day + 31 from SCT), but the patient's respiratory conditions continued to worsen leading to a progressive respiratory distress. A broncho-alveolar lavage performed on day + 34 from SCT showed a high CMV load (50 x 10^3 copies/ml) and the last chest CT scan on day +34 from SCT revealed a worsening of the air-space consolidation suggesting diffuse alveolar damage **(Figure 1C)**.

The patient died on day + 38 from SCT for respiratory failure.

DISCUSSION

Viral infections and in particular CMV infection continue to remain a major problem following allogeneic HSCT with a high rate of morbidity and mortality (7, 12). The pre-transplantation CMV serostatus of the donor and/or recipient remains an important risk factor for post transplantation outcome, despite the use of antiviral prophylaxis and preemptive therapy (4, 7). Primary infection in recipients who are CMV seronegative (R-) and receive a transplant from a CMV seronegative donor (D-) is an extremely rare event (1 to 4%) and no detailed

description have been provided in the literature, to our knowledge, on the few cases reported in the context of case series.

In this paper we report a case of primary CMV infection complicated by a fatal pneumonia probably caused by CMV. The source of the infection remain unknown, but the major hypothesis are the infection from a blood component carrying a low load of CMV positivity or an environmental hospital-acquired infection.

The CMV pneumonia mortality rate is reported up to 90% in patients who have undergone HSCT (13). Definitive diagnosis of CMV pneumonia relies on the detection of the virus in lung tissue; however, lung biopsies are rarely performed in patients with cancer because of the high rate morbidity (e.g. bleeding, bacterial overinfections) associated with this procedure (13). Even if a bioptic/autoptic lung finding of CMV is lacking in our patient, we believe, for the strong clinical impression, the high CMV DNAemia detected and the negative researches for all the other tested microbiological agents, that CMV played a determinant role in the severe lung damage that led to the fatal outcome of the patient.

In order to better manage a primary CMV infection during aplasia and to try to modify its poor prognosis, we here analyse some of the strategies usually followed by HSCT Clinical Units and propose some general recommendations that should be taken into account from bone marrow transplantation centres. Indeed, some of the following points were adopted in our Institution following the reported outcome of this pediatric patient.

There is a general consensus that available strategies to prevent a primary infection in a CMV seronegative recipient should aim to transplant from a CMV seronegative donor and to transfuse exclusively with CMV seronegative or leukocyte-depleted blood products (5, 6, 8, 14).

In order to reduce the risk of CMV transmission from the donor, one could envisage to test the donor as close as possible to the donation and to include CMV PCR, which is more sensitive than an ELISA test to detect an active infection.

To further decrease the probability of CMV infection from blood products, these blood components could also be tested for CMV by PCR, but this strategy would be too demanding from the costs-benefits point of view.

Another option could be to test by CMV specific T cell response (interferon-gamma enzyme-linked immunospot assay ELISPOT), in accordance with the microbiological screening tests recently performed in the solid organs donors and recipients (15). This test would be more sensitive to detect an infection in the window-phase in the donor, and it could be used in selected cases also in the recipient after immune reconstitution. Moreover, the immunologic monitoring of virus-specific T cell recovery may be helpful in determining the therapeutic strategy and identifying the group of patients who are at risk of late-onset CMV disease. Indeed, several studies proved that the recovery of CMV specific CD4+ and CD8+ cells is associated with long-term protection from CMV reactivation and disease and reduced impact of CMV indirect effects (15). Prospective studies are needed to evaluate this monitoring test and its use before transplantation and, eventually, in the high risk phase (<100 days) after HSCT in patients at risk of CMV disease (16).

Prophylaxis with GCV prevents the risk of early CMV infection and disease, but does not improve survival and at the same time may delay specific immune reconstitution, and consequently favour the occurrence of late CMV infection (13, 14). In a CMV negative couple recipient (R) /donor (D), anti-CMV pharmacological prophylaxis is curently not recommended (17), being this strategy usually reserved for patients with high-risk of CMV disease (14). In this setting, one possibility could be to introduce CMV prophylaxis even in negative R/D pairs, to decrease the risk of a primary CMV infection.

Nowadays there are no fair guidelines about the CMV time-monitoring of the recipient in the early post-transplantation setting, but a common strategy is to test CMV DNA once a week or every 2 weeks. In case of high-risk patients, CMV monitoring twice a week is suggested during the period at risk (at least until day +100), in order to initiate promptly pre-emptive treatment (14). Based on our experience, and considering the importance of detecting CMV infection at a very early stage, it should be deserved a more frequent surveillance also in negative R/D pairs. Therefore, in our Centre we revised the internal policy of CMV surveillance introducing a twice a week CMV PCR detection in the first month after transplant also in negative R/D pairs.

Another open question concerns how to treat primary CMV primary infection in a CMV seronegative recipient. Data report the same efficacy of Foscarnet (90 mg/kg/day) and GCV (10 mg/kg/day) on treatment of CMV reactivation (14, 18). Most bone marrow transplantation Centres use GCV as first-line antiviral therapy, but in several studies and in the clinical practice even Foscarnet is administered as first line treatement and sometimes these therapies are not mutually exclusive (14, 19). The use of CMV-specific intravenous immune globulins in addition to antiviral therapy remains controversial, but evidence of reduction in mortality rates of patients with combined therapy versus patients on antiviral monotherapy has been provided (19). Current EBMT guidelines give no indications about the choice of GCV or Foscarnet as preemptive or first line treatment in primary CMV infection or disease (14). In the case of our patient, we preferred to start with Foscarnet (180 mg/kg/day) to avoid an impact on the engraftment and we switched later to GCV (10 mg/kg/day), but this was not effective in resolving the CMV infection. We also used anti-CMV hyperimmune gammaglobulin (Cytotect®), although there is currently no evidence of their efficacy in primary infection. On the basis of the poor outcome observed in our patients, we propose, when a first infection or a severe reactivation of CMV infection do occur, to start with double antiviral treatment, despite the double treatment toxicity, due to the dramatic outcome in the great part of the patients affected. Randomized controlled trials comparing CMV

monotherapy with Foscarnet or GCV versus double therapy have not been reported, due to the very high mortality rate in these cases. Nevertheless, more studies are needed to outline the best treatment of CMV primary infection and the safety profile of the double therapy.

Several trials have proven the efficacy of anti-viral cytotoxic T-cells (CTL) for the treatment of viral infections in patients after HSCT (20-22), and this strategy can be enlisted among the therapeutic options to be considered for patients with life threatening infections in which primary treatment strategies, such as anti-viral therapy, fail. Nevertheless, the logistics, cost, and labor intensity involved in generating histocompatible virus-specific CTLs remain challenging as well as it remains a relatively restricted array of CMV T cell epitopes in a given individual (23).

A recent study showed the possibility to customize anti-CD3 activated T cells (ATCs) armed with chemically heteroconjugated anti-CD3 x polyclonal anti-CMV bispecific antibody (CMVBi), using cryopreserved HLA-identical donor T cells or unrelated donor T cells. This approach could be used to target the broadly relevant epitopes expressed on the CMV-infected cells and is likely to be texted in the next future in phase I/II clinical trials to treat patients affected by CMV infections in allogeneic SCT or organ transplantation recipients (23).

In conclusion, the possibility of CMV infection in a CMV negative (R/D) pair should not be underestimated for the severe and frequent fatal consequences, and new strategies to prevent and to treat this type of occurrence should be evaluated in bone marrow transplantation centres.

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FIGURE 1 (A, B, C) LEGEND: Evolution of CT scan images during primary CMV infection complicated with CMV pneumonia after haploidentical bone marrow transplantation.

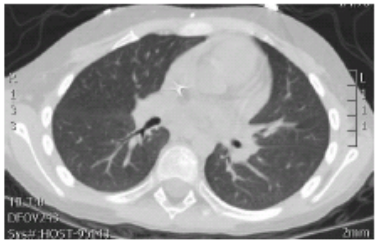
A: day + 13 after HSCT. No focal pleuro-parenchymal alterations in both the lungs.

B: day + 25 after HSCT. Some peri-hilar bilateral parenchymal thickening, with air bronchograms and confluence appearance. Increased parenchymal density with "ground-glass" appearance in both the lungs. In the spared regions, it is evident a reticular appearance, as for interstitial oedema. One cm pericardial effusion flap.

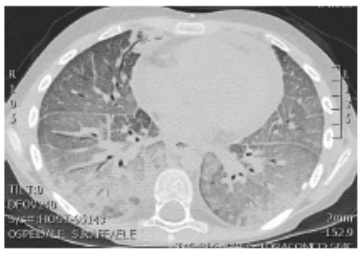
C: day + 34 after HSCT. Increase in parenchymal density in the sites of "ground-glass" alteration, with extended areas of consolidation. Some spared subpleural areas. No pleural effusion. Unchanged flap of pericardial effusion.

FIGURE 1 (A, B, C): Evolution of CT scan images during primary CMV infection complicated with CMV pneumonia after haploidentical bone marrow transplantation.

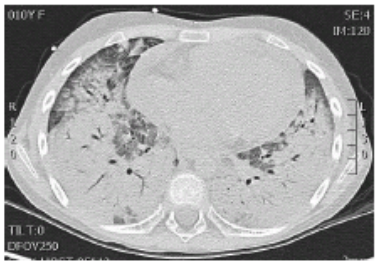
A: day + 13 after HSCT



B: day + 25 after HSCT



C: day + 34 after HSCT



INVITED COMMENTARY

Management of Autoimmune Hepatitis in Children: How Many Steps Away From Common Agreement?

*Maria Pia Cicalese and †Raffaele Iorio

See "Autoimmune Hepatitis in Children in Eastern Denmark" by Vitfell-Pedersen et al on page 376.

Autoimmune hepatitis (AIH) may be particularly aggressive in children and induce severe liver damage, if untreated. On the contrary, immunosuppressive therapy, with prednisolone alone or in combination with azathioprine, leads to remission in 80% of cases resulting in a normal life expectancy, good quality of life (1), and a transplant-free survival rate of 90% (2).

In this issue of *JPGN*, Vitfell-Pedersen et al report the features and long-term outcome of children with AIH enrolled in a paediatric population-based survey from the eastern part of Denmark (3). This retrospective study, if compared with previous European series, shows some unusual findings such as male preponderance, extremely low rate of anti-liver kidney microsomal type 1 antibody–positive patients, and absence of fulminant cases (4,5). Despite these peculiarities, which could be due to bias of the study, it is noteworthy that liver cirrhosis, present in 69.6% of patients at diagnosis, did not affect either the overall survival or the survival free of liver transplantation.

This favourable outcome could suggest the possibility, reported in the literature, but still a matter of debate among experts, of reversibility of liver fibrosis in some chronic liver diseases (6,7). Obviously, this is a simple hypothesis because histological evaluation of the liver was not performed in Danish patients with sustained biochemical remission.

Another relevant finding of the Danish study is that 84% of patients achieved remission in a median of 5.4 months from the start of treatment (60.6% on treatment with prednisolone and azathioprine and 24% on prednisolone alone). Consequently, treatment with prednisolone and azathioprine seemed to be more effective than prednisolone alone in inducing remission. Unfortunately, the authors did not clarify why a single treatment schedule was not used and azathioprine added only to some patients (poor control of transaminases, steroid adverse effects?) or why so many patients were not controlled by treatment. At the end of follow-up, 15% of the patients were in stable remission and off therapy. Even for this issue, no information about the putative predictors of such a good outcome was provided. Actually, despite the availability of many studies and guidelines for AIH (8), many aspects concerning children with AIH remain unclear. In this context, it would have been useful to have more details about clinical features of patients treated with steroid alone versus those treated with steroid and azathioprine, doses of immunosuppressant drugs during the different phases of the treatment, duration of each phase, and minimum required doses to maintain remission to draw conclusions about the best mode of treatment for AIH in children.

Other limitations of the study are represented by the scarce use of the available autoantibody repertoire for the diagnosis of AIH (8,9) absence of indication of the autoantibodies' serum levels considered positive for children (8), and limited use of cholangiography, which was not performed in all children with AIH to exclude biliary involvement according to American Association for the Study of Liver Diseases guidelines (8).

Even with these limitations, we think that Vitfell-Pedersen study is valuable because it draws attention to many unclarified points of AIH in childhood. To date, because of the relative rarity of this condition, in common clinical practice the management of children with AIH often reflects the preferences of individual centres (8). It is probably time to initiate large multicentre prospective studies to reach common agreement on the management of children with AIH.

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From the *Pediatric Immunology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milan, and the †Department of Pediatrics, University of Naples Federico II, Naples, Italy.
Address correspondence and reprint requests to Maria Pia Cicalese, MD, University Federico II of Naples, Naples, Italy (e-mail: mariapia.cicalese@tiscali.it).
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SUMMARY AND FINAL REMARKS

PIDs represent a heterogeneous and growing-in-characterization group of monogenic conditions which display very different phenotypes, that can range from being asymptomatic to manifestation of life-threatening conditions (2, 187).

Among different therapeutic approaches, in recent years GT has attracted attention for the opportunity to provide a definitive, targeted and personalized treatment, with a good safety profile for several PIDs. As a consequence, GT has enlarged the percentage of patients affected by PIDs, for whom a compatible bone marrow donor was not available, that could have been cured. Moreover, new acquisitions gained in this field have created a new groundwork by the deeper study of many immunodeficiencies and their molecular and pathogenetic mechanisms.

As the safety of the patients remains a crucial point, there is great expectation in the patients' community that even safer and more effective approaches can be developed for a wider spectrum of PID and future goals include the conduct of prospective treatment studies to determine optimal therapies for PIDs, and for other genetic diseases.

During my PhD program, I contributed to the development of "New approaches in management and treatment of primary immunodeficiencies and other genetic diseases", in particular of innovative advanced cellular and gene therapy approaches.

Moreover, I contributed to the clinical and biological characterization of patients with immunodeficiencies, in order to clarify unsolved issues and unknown mechanisms underlying the functionality of the immune system, determining a given clinical phenotype.

Another line of research has been the implementation of new approaches for the clinical management of children affected by hematological and immunological disorders undergoing bone marrow transplantation.

Furthermore, I have described the use of new drugs in pediatric population and I have characterized severe and unusual conditions in pediatric population.

Overall, most of my studies were focused on investigating the safety and efficacy of alternative therapeutic strategies for the treatment of PIDs and other genetic

diseases and to implement the standards of care of bone marrow transplantation for pediatric patients with hematological and immunological diseases.

These results represent an important goal in view of a coming era in which gene therapy and other advanced therapies will quickly move from being an experimental approach to a standard cellular therapy.

ABBREVIATIONS

AE	Adverse Event
ADA	Adenosine Deaminase
ADR	Adverse Drug Reaction
AE	Adverse Event
AIEOP	Associazione Italiana Ematologia Oncologia Pediatrica
ALT	Alanine Aminotransferase
AME	Absorption, metabolism, and excretion
ANA	Antinuclear Antibody
AST	Aspartate Aminotransferase
BMT	Bone Marrow Transplant
CD4+	T cell subset
CD34+	Haematopoetic stem progenitor cells
CGD	Chronic Granulomatous Disease
CNS	Central Nervous System
CRF	Case Report Form
CSR	Clinical Study Report
CRT	Catheter-Related Thrombosis
CT	Computed Tomography
CTCAE	Common Terminology Criteria for Adverse Events
CUP	Compassionate Use Programme
CVC	Central Venous Catheter
dADP	Deoxyadenosine diphosphate
dAMP	Deoxyadenosine monophosphate
dATP	Deoxyadenosine triphosphate
dAxP	Deoxyadenosine nucleotides (dAMP, dADP, dATP)
DMD	Duchenne Muscular Dystrophy
ECG	Electrocardiogram
EEG	Electroencephalography

EMA	European Medicines Association
ERT	Enzyme replacement therapy
F-U	Follow-up
G-CSF	Granulocyte Colony Stimulating Factor
GSK	GlaxoSmithKline
GT	Gene Therapy
GTMPs	Gene Therapy Medicinal Products
GVHD	Graft Versus Host Disease
HLH	Hemophagocytic Lymphohistiocytosis
HPV	Human papilloma virus
HSC	Hematopoietic Stem Cell
HSC-GT	Hematopoietic Stem Cell - Gene Therapy
HSR	Hospital San Raffaele
HSCT	Hematopoietic Stem Cell Transplant
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IV	Intravenous
IVIG	Intravenous immunoglobulin
LTFU	Long term follow-up
LV	Lentivirus
MAA	Market Authorization Application
MABs	Mesoangioblasts
MDS	Myelodysplastic syndrome
MLD	Metachromatic Leukodystrophy
MRI	Magnetic Resonance Imaging
NK	Natural Killer
NAFLD	Non-Alcoholic Fatty Liver Disease
NPP	Named Patient Program
PIDs	Primary Immunodeficiencies
PIDTC	Primary Immune Deficiency Treatment Consortium
PCR	Polymerase Chain Reaction

PEG	Polyethylene glycol
PEG-ADA	Polyethylene Glycol-modified bovine Adenosine Deaminase
VEP	Visually Evoked Potentials
RAP	Reporting and Analysis Plan
RBC	Red Blood Cells
RV	Retrovirus
SAE	Serious Adverse Event
SCE	Summary of Clinical Efficacy
SCID	Severe Combined Immunodeficiencies
SCS	Summary of Clinical Safety
SCT	Stem cell transplant
SMQ	Standardized MedDRA Query
SOC	System Organ Class
PT	Preferred term
TIGET	Telethon Institute for Gene Therapy
UTI	Urinary Tract Infection
WAS	Wiskott-Aldrich Syndrome
WBC	White Blood Cell
WG	Working Group
VCN	Vector Copy Number

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3. Long-Term Safety and Efficacy of Retroviral-Mediated Gene Therapy for ADA-SCID.
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4. Persistent Multilineage Engraftment and WASP Restored Expression After Lentiviral Mediated CD34+Cells Gene Therapy for the Treatment of Wiskott-Aldrich Syndrome.
   By: Scaramuzza, S.; Giannelli, S.; Ferrua, F.; et al.
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5. Persistent multilineage engraftment and WASP expression after lentiviral mediated CD34+cell gene therapy for the treatment of Wiskott-Aldrich Syndrome
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12. The nursing management of ADA-SCID children treated with gene therapy
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EUROPEAN CURRICULUM VITAE FORMAT

PERSONAL INFORMATION

Name	**Maria Pia Cicalese**
Address	Residence: Via Ruocco, 6 - 80016 Marano di Napoli (NA) – Italy Office: via Olgettina 60, 20132 Milano - Italy
Telephone	Office : +390226436327 Personal Mobile: +393475764974
Fax	Office : +390226436545
E-mail	mariapia.cicalese@tiscali.it cicalese.mariapia@hsr.it
Nationality	Italian
Date of birth	15th March, 1980

WORK EXPERIENCE

• Dates (from – to)	April 2011-to date
• Name and address of employer	San Raffaele Hospital, Milano
• Type of business or sector	Paediatric Immuno-haematology and Bone Marrow Transplantation Unit
• Occupation or position held	Staff Physician
• Other positions held	From 2013 to date, Transplant Coordinator and Quality Manager for Paediatric Immuno-haematology and Bone Marrow Transplantation Unit
• Main activities and responsibilities	Staff Physician in the Paediatric Immunology and Bone Marrow Transplantation Unit, gaining experience and skills in autologous and allogeneic bone marrow transplantation in adults and children; Responsible for the Pediatric Immunohaematology Outpatient Clinic; Scientific collaborator of several Phase I-II clinical trials of genetic and cellular therapy.
• Dates (from – to)	March 2010-April 2011
• Name and address of employer	San Raffaele Hospital, Milano
• Type of business or sector	Paediatric Immuno-haematology and Bone Marrow Transplantation Unit
• Occupation or position held	Clinical Fellow

• Main activities and responsibilities	Clinical Fellow in the Paediatric Immunology and Bone Marrow Transplantation Unit, gaining experience and skills in autologous and allogeneic bone marrow transplantation in adults and children; Responsible for the Pediatric Immunohaematology Outpatient Clinic; Scientific collaborator of several Phase I-II clinical trial of genetic and cellular therapy.

Education and training

• Dates (from – to)	January 22nd 2010: Specialization in Paediatrics with honour.
• Name and type of organisation providing education and training	School of Paediatrics at the University Medical School of Naples "Federico II".
• Title of qualification awarded	Specialist in Paediatric
• Dates (from – to)	November 2008-June 2009
• Name and type of organisation providing education and training	Visiting Paediatrician at Paediatric Liver Unit King's College Hospital, London, UK.
• Principal subjects/occupational skills covered	Clinical skills on Liver Transplantation in children in a Third Level Centre, clinical research experience.
• Dates (from – to)	2005-2010
• Name and type of organisation providing education and training	Resident in Pediatrics at the School of Pediatrics of the University Medical School of Naples "Federico II".
• Principal subjects/occupational skills covered	Attendance of the Units of General Paediatric, Gastroenterology (Inflammatory Bowel Disease, Celiac disease, Cystic Fibrosis…), Hemato-Oncology, Intensive Neonatal Care Unit, Infectious Disease, Paediatric Endocrinology, Immunology and Immunodeficiency, Hepatology. Involvement in clinical research projects at Liver Unit of Department of Paediatrics of the University Medical School of Naples "Federico II" and research experience in the fields of Clinical Nutrition, Enteral and Parenteral Nutrition in patients with Short Bowel Syndrome and other forms of Intestinal Failure, Obesity and Metabolic Syndrome in childhood.
Dates	February 2005: Medical Licence.
Name and type of	University Medical School of Naples "Federico II" - Italy

organisation providing education and training	
Dates	28th September, 2004
Title of qualification awarded	Medical Degree: 110/110 with honours Title: "Smith-Lemli Opitz Syndrome: study of a case series"
Name and type of organisation providing education and training	University Medical School of Naples "Federico II" - Italy
Dates	10th June, 2004
Title of qualification awarded	"Basic Life Support": BLS. Retraining in 2010.
Name and type of organisation providing education and training	Italian Peadiatric Society (SIP) and Italian Society of Paediatric Emergency (SIMEUP). Retraining in San Raffaele Hospital, Milan.
Dates	September 2009
Title of qualification awarded	"Paediatric Basic Life Support": PBLS. "Paediatric Advanced Life Support": PALS. Retraining of PBLS in 2010.
Name and type of organisation providing education and training	Italian Resuscitation Council (IRC) and Italian Society of Paediatric Emergency (SIMEUP). Retrainingof PBLS in San Raffaele Hospital, Milan.

PERSONAL SKILLS AND COMPETENCES

MOTHER TONGUE	Italian
OTHER LANGUAGES	
	English
• Reading skills	excellent
• Writing skills	excellent
• Verbal skills	good
	Spanish
• Reading skills	basic
• Writing skills	basic
• Verbal skills	basic

SOCIAL SKILLS AND COMPETENCES *(for example culture and sports), etc.*	Skilled in clinical and technical management of acute and chronic sick children. Able to deal with paediatric patients from newborn to adolescents. Skilled in family counselling and parents' psychological support. Leading and mentoring junior medicine students. Endowed with a flair for working in team. Living with other people, I lived even in multicultural environments, with good communication and cooperation skills.
ORGANISATIONAL SKILLS AND COMPETENCES	Coordination and administration of people, research projects and budgets at work.
TECHNICAL SKILLS AND COMPETENCES	Skilled in the use of word, excel, power point and access and in the use of database and Internet. Basic statistic knowledge.
Driving licence(s)	Driving licence B
Other Activities	reading, photographing, travelling, trekking, snorkelling. volunteering and travelling in Europe and outside Europe: Africa, Russia, Mexico, USA. playing guitar
REFEREES	Prof. Alessandro Aiuti Associate Professor University of Tor Vergata Coordinator of Clinical Research San Raffaele Hospital Via Olgettina, 60 20132 Milano, Italy aiuti.alessandro@hsr.it Dott. Fabio Ciceri Director Department of Haematology and Bone Marrow Transplantation Unit – San Raffaele Hospital Via Olgettina, 60 20132 Milano, Italy ciceri.fabio@hsr.it

Date 30/03/2015 Signature: Maria Pia Cicalese