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Director
Prof. Claudio Pignata

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New characterization of congenital immunodeficiencies due to different functional alterations

Student
Dr. Anna Fusco

Tutor
Prof. Claudio Pignata

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INDEX

Background and Aim of the Project

Primary immunodeficiency diseases are heritable disorders of immune system function [1]. Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness. Apart from physical barriers, the immune response is composed from a diverse network of defenses, including cellular components and soluble mediators. A proper normal immune response relies on the innate immune response that mounts rapid and nonspecific responses to initial infections and later on the adaptive response characterized by specific responses to a particular pathogen. The innate immune response involves three major cell types: phagocytic cells, such as neutrophils and macrophages, natural killer (NK) cells and antigen-presenting cells, which are also involved in the induction of an adaptive immune response. The adaptive immune system includes T and B lymphocytes and thus it can be divided into cellular and humoral responses. However, these components of immune system work to maintain normal host function and resistance to infection in a well orchestrated and integrated unique system.

In the last 5 decades since the first human host defect was identified, more than 200 primary immunodeficiency (PID) syndromes have been described. PIDs can be divided into subgroups based on the component of the immune system that is predominantly affected, including T, B, NK lymphocytes, phagocytic cells and complement proteins [2]. Diseases in which lymphocyte functions are predominantly affected are commonly divided into three main groups. The antibody deficiencies (B-cell or humoral immunodeficiencies), characterized by a genetic lesion that selectively affects antibody production, but a normal cell-mediated immunity; the cellular deficiencies, in which antibody production is largely normal, whereas cellular effector mechanisms are compromised; and the combined

immunodeficiencies, characterized by impairment of both effector arms of specific immunity.

T-lymphocyte and B-lymphocyte responses are not independent of one another; for example, B cells can activate antigen-specific T cells for a cellular immune response, while an efficient B-cell antibody response depends in part on T-cell activation of B lymphocytes. Thus, defects in either cell type have the potential to affect both cellular and humoral immunity to varying degrees.

The last category contains the subgroup of severe combined immunodeficiency (SCID) disorders consisting of genetically determined blocks in the T-lymphocyte differentiation program. The intrinsic impairment of T-cell development is variably associated with defective differentiation of other hematopoietic cell lineages. In the absence of mature T cells, adaptive immunity is abrogated, resulting in a broad-spectrum susceptibility to multiple pathogens, among which a number of opportunistic microorganisms predominate. In these cases, the diagnosis will most often be considered when infections are considered to be more frequent or severe, unusually resistant to standard therapies, or caused by unusual (opportunistic) organisms [1]. In fact, affected patients often present early in life with failure to thrive and disseminated infections. Thus, SCIDs represent a group of diverse genetic disease affecting the immune system that share common clinical features including overwhelming viral and fungal infections.

As a matter of fact, the discovery of more than 200 distinct clinical entities which differ in either the genetic cause or the altered immunological function led to an uncomparable increase in the knowledge of the intimate mechanism by which a proper immune response is generated. Intriguingly, most of the genes whose alterations underlie PID are selectively expressed in hematopoietic cells with a few exception as, for example, Ataxia Telangiectasia Mutated (ATM) gene, also expressed in Purkinje cells, and

Adenosine Deaminase (ADA) which is ubiquitous. This peculiarity leads to the increased susceptibility to infections, which represents the most remarkable presenting sign of immunodeficiencies. This dogma, however, led to underestimate those novel immunodeficiencies, which have different features involving other non-hematopoietic tissues.

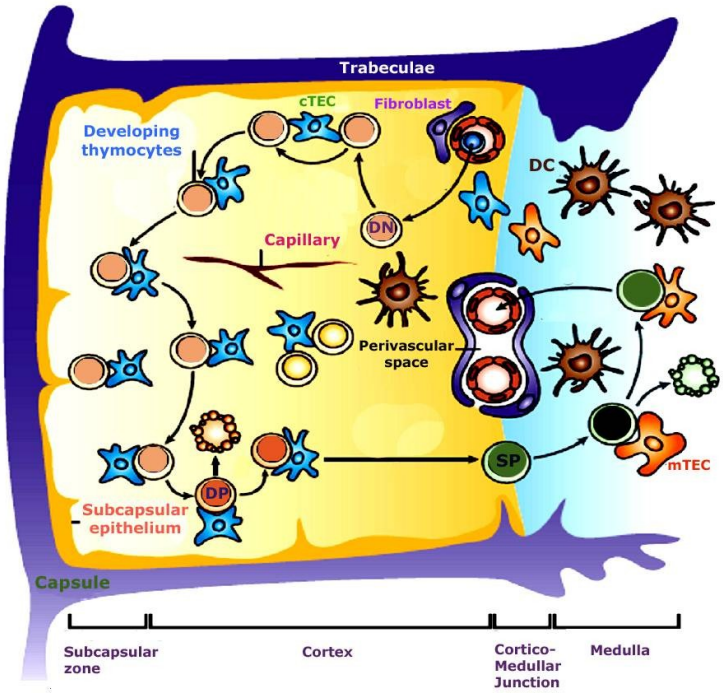
In the 1996, for the first time, a novel form of SCID (MIM 601705; Pignata guarino syndrome) was described. It represents the human equivalent of the well known murine phenotype defined as Nude/SCID [3] and it is the first example of SCID not primarily related to an hematopoietic cell abnormality, but rather to thymic aplasia [4]. Moreover, the hallmark of the Nude/SCID phenotype is congenital alopecia, from which the "Nude" term for the murine equivalent derived [5], always associated with a profound T-cell defect. Thus, because of these atypical presenting signs, different from the clinical signs of the "classical" forms of SCID, the human Nude/SCID was identified only 30 years after the first description of the Nude phenotype in mice by Flanagan.

In keeping with these observations, the aim of this thesis was to characterize some immunodeficiencies associated to different functional alterations in order to better understand the mechanisms involved in the pathogenesis of such immune diseases. The results of this thesis by adding novel information in this field could be useful both in the clinical practice and in the basic research of immune disorders.

CHAPTER I

Immunodeficiencies due to alteration of the thymic function

In humans, lymphopoiesis occurs in bone marrow and thymus. In particular, B lymphocytes mature within the bone marrow while T precursor cells leave the bone marrow and enter the thymus which is the only organ capable to support the differentiation and selection of mature T lymphocytes [6]. The thymus-dependent development of T cells requires that the developing thymocytes and thymic stromal cells have to communicate with each other in close proximity and remotely. In fact, thymocyte development is based on a stringent repertoire selection after which only 1-3% of thymocytes can be exported from the thymus [7, 8].



Moreover, developing thymocytes by coaching thymic stromal cells, provide the appropriate microenvironment necessary to promote and regulate further thymocytes development [9]. Therefore, the lympho-stromal communication is a bilateral coordination, or crosstalk, between architectural stromal cells and travelling thymocytes [10]. The thymic stromal compartment consists of several cell types that collectively enable the attraction, survival, expansion, migration, and differentiation of T-cell precursors. The thymic epithelial cells (TECs) constitute the most abundant cell type of the thymic microenvironment and can be differentiated into morphologically, phenotypically, and functionally separate subpopulations of the postnatal thymus [11]. The epithelial compartment of the thymus originates from the endodermal layer of the anterior foregut [12]. During pharyngeal pouch development, these cells delaminate from the inner layer of the foregut and grow into the underlying mesenchymal tissue compartment that is of neural crest origin. Expression analyses of the transcription factor Foxn1 have identified the ventral aspect of the third pharyngeal pouch endoderm as the only site from where the thymus primordium emerges [13]. The early organogenesis of the thymus is closely coupled to that of the parathyroid gland, which has its origin in the dorsal part of the third pharyngeal pouch [11]. It begins to express Foxn1 at about E11.5, in mice. The Foxn1 gene is expressed in skin epithelial cells, hair follicles, and all subpopulations of thymic epithelial cells [14, 15]. In its function as a transcriptional activator, Foxn1 is absolutely required for the normal differentiation of hair follicles and thymus epithelial cells [5, 11, 16]. Foxn1 encodes a transcription factor whose function is essential for subsequent epithelial differentiation; without it, colonization of the anlage by thymocyte progenitors fails [17] and thymopoiesis is aborted, resulting in severe immunodeficiency [18]. In fact, both mice with a recessive mutation of

Foxn1 lacking the DNA-binding domain and humans with a nonsense mutation resulting in a complete absence of a Foxn1 protein display the 'nude' phenotype, which is characterized by hairlessness and congenital athymia. Specifically, these Foxn1 mutations result in defective hair differentiation, although the hair follicles are normally specified and hair development is correctly initiated. The formation of a thymus primordium in nude mice is normal up to the point when hematopoietic progenitors enter the wild-type thymus. Importantly, neither the formation of the third pharyngeal pouch nor the patterning of the thymus primordium is affected by the lack of Foxn1 [18]. However, the epithelial cells of the nude anlage lack the ability to induce the entrance of hematopoietic progenitor cells into the epithelial cluster and thus preclude the generation of thymocytes [19]. It is still unknown which signal(s) determine(s) the site and also the size of the normal thymic anlage and whether these or other signals initiate Foxn1 expression. Clearly, genetic abnormalities involving the formation of pharyngeal pouches (such as deficiencies in Tbx1, Pbx1, Eya1, Six1) also have an impact on the thymus formation [20-22]. The most studied one is the gene encoding for the transcription factor Tbx1 involved in the development of the pharyngeal apparatus and its derivates [23-26]. Tbx1 belongs to the family of T-box transcription factors that are characterized by a common DNA-binding sequence, designated T-box, and the capacity to form combinatorial interactions with other transcriptional regulators [27]. A specific role for Tbx1 in thymus development has initially been inferred from a characteristic expression pattern in both the third pharyngeal pouch endoderm and the adjacent mesenchyme. By contrast, Tbx1 expression is excluded from neural crest cells and ectomesenchyme [11]. The homozygous loss of Tbx1 causes thymic hypoplasia and other severe pathologies affecting the derivates of the third and fourth pharyngeal arches, and these developmental anomalies are indistinguishable from changes observed after the deletion of premigratory

neural crest cells [23-26]. Interestingly, mice heterozygous for a null allele of Tbx1 demonstrate only a mild phenotype without thymus anomalies, while Tbx1-deficient mice transgenic for a bacterial artificial chromosome containing the human TBX1 display (among other anomalies) thymic hypoplasia or aplasia [28]. This result argues for a gene dosage effect of Tbx1 in the pathogenesis of DiGeorge syndrome.

Briefly, the journey through the thymus began when committed lymphoid progenitor cell (CD34+) arise in the bone marrow and, via the blood, enters the thymus at the cortico-medullary junction [6]. In this phase, the progenitor cell not expresses the glycoprotein CD1a, which, instead, will be express by intrathymic lymphocytes [29]. In humans, this event occurs at the eighth week of gestation [30]. In the thymus, this cell lose the potential for B-cell and Natural-Killer (NK) cell development [31] resulting in a double negative (DN; no CD4 or CD8), committed T-cell precursor. On the basis of the expression profiles of CD25 and CD44 showed by DN thymocytes during their maturation process it's possible discriminate four sequential stages of differentiation, such as: DN1, CD44+CD25-; DN2, CD44+CD25+; DN3, CD44-CD25+; DN4, CD44-CD25- [31]. Along this developmental pathway, immature DN thymocytes promote the differentiation of thymic stromal cells and trigger the formation of the cortical-epithelial environment in the thymus [32-35]. Therefore, the differentiation of thymocytes from the DN1 stage to the DN3 stage (CD4-CD8-CD25+CD44-) regulate the differentiation of thymic epithelial cells (TECs) into cortical TECs (cTECs) that form the cortical environment in the thymus. Concomitantly, DN thymocytes from the cortico-medullary junction migrate to the cortex where the maturation process began [6]. DN1 and DN2 lymphocytes can potentially give rise to either $\alpha\beta$ or $\gamma\delta$. The first TCR chains that are expressed early on thymocytes surface are the δ and γ chain; later, β chain is rearranged and the last one is the α chain. The cells that

proceed along the αβ TCR pathway are the DN3 cells that first express pre-TCR-α (pTα) that pairs with the TCR β–chain which is the product of a set of somatic DNA rearrangements that require expression of Recombination-Activating gene (RAG1) and RAG2 proteins. This is the first checkpoint (β-selection) of T-cell development to the DN3 and DN4 stages. In association with the pre-TCR αβ the CD3/ζ complex is expressed at the cell surface to mediate signal transduction within the cell. T cells that emerge from β-selection (late DN3 and DN4) undergo to recombination at the TCR a locus producing the second component chain of of the mature ab antigen receptor. During this stage the thymocytes begin to express co-receptor proteins, most often CD8 first, followed by CD4. Eventually, a large population of double-positive (DP; CD4+CD8+) αβ-TCR-expressing immature cells is formed. Later, only a small aliquot of these DP cells with a functional rearranged TCR shall migrate into periphery [31]. Within the thymus, DP thymocytes undergo to positive and negative selection leading to single positive (SP) cells, CD4+CD8- or CD4-CD8+, that migrate to medulla. In this thymic compartment the thymocytes began to express the homing receptor CD62L, a characteristic molecule of naïve cells. Finally, they leave the thymus as mature naïve lymphocytes. This population is chacarcterized by the expression of the RA isoform of the CD45 molecule, derived from the alternative splicing. The isoform CD45RO is specifically of memory cells [36]. Later, it was proposed the combination use of different markers for naïve cells, such as CD62L and CCR7 [37]. In the newborn, the most of peripheral T cells (90%) shows a naïve phenotype, progressively decreasing during infancy (60%) and adulthood, with a paralleling increase of memory lymphocytes [38]. Thus the thymus is considered needful for the maturation of naïve cell, expecially for the CD4+ naïve cells. Thus, the analysis of this cell subset could be useful to evaluate thymic output and subsequently to identify diseases due to abnormality of thymic function.

§ 1.1 The Nude/SCID phenotype as real human model of athymia

More than 30 years from the original description of spontaneous murine disease, the first human equivalent of the Nude/SCID phenotype was described in 1996. The identification of the human counterpart of nude mouse phenotype began with identification of two sisters, whose clinical phenotype was characterized by congenital alopecia, eyebrows, eyelashes, nail dystrophy and a severe T-cell immunodeficiency, inherited as an autosomal-recessive disorder [3]. The T-cell defect was characterized by severe functional impairment, as shown by the lack of proliferative response. The functional abnormality occurred in spite of the presence of a few phenotypically mature T cells, thus suggesting the qualitative nature of the defect [3]. Since alopecia and nail dystrophy are also found in other syndromes, such as dyskeratosis congenita (DC) [39, 40], other signs of this disease were investigated. Differently from DC, major signs, such as abnormal pigmentation of the skin and mucosal leucoplakia, were lacking in Nude/SCID and the immunological abnormalities were different from those reported in patients with DC in both severity of clinical course and type of alterations [41, 42]. Moreover, both patients showed alopecia at birth and in one sib it still persisted after bone marrow transplantation, thus ruling out that it was secondary to an acquired skin damage. This finding suggested that the alopecia in this patient was primitive in nature [3]. Furthermore, these features were similar to those reported in athymic mice, that completely lack body hair and in which restoration of a thymus did not lead to hair growth [43]. Taken together, these observations suggested an association between alopecia and the immunodeficiency reported in the 2 sisters, linked to a single gene defect [3].

In humans, "*FOXN1*" is located on chromosome 17 [44] and it encodes a transcription factor mainly expressed in the epithelial cells of the skin and

thymus, maintaining the balance between growth and differentiation. Later, a molecular analysis in these patients was performed and revealed the presence of a C-to-T shift at 792 nucleotide position in the sequence of the *FOXN1* cDNA, that leads to a nonsense mutation R255X in exon 4 (formerly exon 5), with a complete absence of a functional protein similar to the previously described rat and mouse *Foxn1* mutations [45-47].

Since the first description of these Nude/SCID patients other patients with a similar phenotype were identified. In particular, a Nude/SCID patient was diagnosed in Portugal. The newborn presented with alopecia and nail dystrophy associated with severe infections. The screening for R255X mutation of *FOXN1* gene revealed that the patient was homozygous for the mutation. It should be noted that the patient was born to consanguineous parents, both from Lisbon. (communicated to European Society for Immunodeficiencies, 2006)

Moreover, the 2 sisters in whom this mutation was found at the homozygous status, were born to consanguineous parents originating from a small community in southern Italy. In this village additional patients of previous generations were affected with congenital alopecia and died early in childhood because of severe infections [48]. In order to identify an ancestral founder effect for this phenotype, in this population, that can be considered isolated for the geographical location of the village, a genetic screening for the presence of the *FOXN1* R255X mutation was performed. The study led to identify 55 subjects, corresponding to 6.52% of the studied population, who carried the mutation in heterozygousity [48]. All the affected cases belonged to an extended 7-generational-pedigree, founded by a single ancestral couple born at the beginning of the 19th century from which four family groups originated. The pedigree analysis revealed that 33.3% of heterozygotes inherited the mutant allele from their mother, whereas 66.7% from their father. Moreover, this pedigree was also characterized by a high

rate of consanguineous matings, typical of small community; in fact, 14 out of 151 marriages were between consanguineous [48].

The identified heterozygous subjects were examinated with particular regard to ectodermal alterations of hair and nails, in order to define whether the heterozygosity was associated with mild clinical signs. The examination revealed no association between gross alteration of the hair and heterozygousity, while 39 of the 55 heterozygous subjects showed a nail dystrophy. Of note, this alteration was not found in other control subjects and was not related to an acquired form of nail dystrophy. The most frequent phenotypic alteration affecting the nails was koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; the less frequent, canaliform dystrophy and a transverse groove of the nail plate (Beau line) [49]. However, the most specific phenotypic alteration was leukonychia, characterized by a typical arcifom pattern resembled to a half-moon and involving the proximal part of the nail plate. These alterations of digits and nails were also reported in a few strains of nude mice. *FOXN1* is known to be selectively expressed in the nail matrix where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygousity for this molecular alteration [49].

Later, the identification of the haplotype for the *FOXN1* locus, by analysing 47 chromosomes carrying the mutation R255X, led to identify the single ancestral event that underlies the human Nude/SCID phenotype. As this form of SCID is more severe due to the absence of the thymus, a screening program for prenatal diagnosis in this population was considered most important for the identification of fetuses carrying the mutation. During the screening program in this population a high rate of mortality in the first

trimester was also found. This event was not justified by SCID *per se* thus suggesting the involvement of an unknown mechanism that caused mortality.

The genetic counselling offered to couples at risk through the prenatal diagnosis by direct genetic analysis of *FOXN1* led to identify an affected female fetus during the first trimester of pregnancy, thus indicating the importance of this effort. The fetus was homozygous for R255X mutation and the autoptical examination revealed the absence of the thymus and a grossly abnormal skin which was tighter than usual and which showed basal hyperplasia and dysmaturity, suggestive of an impaired differentiation program. These signs confirmed that FOXN1 is involved in thymic development and skin differentiation in humans. Of note, the fetus also showed multiple-site neural tube defects, including anencephaly and spina bifida that could explain the high rate of mortality in utero observed in the described population. Another aspect that led to consider the Nude/SCID mutation and anencephaly causally related is the fact that the other forms of SCID become clinically evident in post-natal life, when the protection of the newborn by the mother immune system declines. Moreover, there was an evidence that the mouse Foxn1 gene is also expressed in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain [50]. Further studies on these aspects are performed on a second female fetus affected with Nude/SCID phenotype and belonging to the same family of the previous fetus. She was examined for the presence of CNS developmental anomalies. In particular, the abdominal sonography, revealed a morphologically normal brain, apart from a probable absence of the septum pellucidum, as suggested by failure to visualize cavum septi pellucidi (CSP) by means of ultrasound, in that only the medial wall of the frontal horn could be detected. At this gestational age, CSP is a fluid-filled cavity located in between the two layers of the septum pellucidum. In the Nude/SCID fetus, autopsy failed to reveal any macroscopic abnormality

of brain structures, including choroid plexus, except the abnormality in the development of corpus callosum.

Thus, these findings suggest that FOXN1 may also be implicated as co-factor in the development of vital systems required for a proper fetus development, thus explaining the mortality in the first trimester, not justified by the SCID *per se*.

These data have been published in a chapter of the book entitled *Forkhead Transcription Factors: Vital Elements in Biology and Medicine*, in *Rivista Italiana di Genetica e Immunologia Pediatrica* and in *Journal of the Neurological Sciences.* (See below for the manuscripts).

Human Clinical Phenotype Associated with *FOXN1* Mutations

Claudio Pignata,* Anna Fusco and Stefania Amorosi

Abstract

In *humans*, a proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. Disruption of any part of the orchestrated immune response results in the inability to control infections and, subsequently, in illness. An impairment of both effector arms of the specific immunity characterizes the clinical phenotype, known as severe combined immunodeficiency (SCID), which represents a heterogeneous group of inherited disorders due to abnormalities of T, B and NK cells. The first congenital SCID was described as spontaneous immunodeficiency in 1966 in mice and referred as Nude/SCID, based on the association of athymia with complete hairless. In 1996, the human equivalent of the murine Nude/SCID phenotype (MIM #601705) was reported. As in mice, also in *humans* this form is characterized by an intrinsic defect of the thymus, congenital alopecia and nail dystrophy and is due to mutations of the *FOXN1* gene, as well. FOXN1 is mainly expressed in the thymus and skin epithelial cells, where it plays a critical role in differentiation and survival. *FOXN1* belongs to the forkhead box (FOX) gene family that comprises a diverse group of 'winged helix' transcription factors involved in development, metabolism, cancer and aging. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions. In immune system, alterations of FOXN1 result in a thymus anlage that lacks the capacity to generate mature and functional thymocytes. Because the significant expression levels of FOXN1 in skin elements, keratinocytes have been successfully used to support a full process of human T-cell development in vitro, resulting in the generation of mature T-cells from hematopoietic precursor cells (HPCs). This finding would imply a role for skin as a primary lymphoid organ. Thus, the present chapter will focus on the information that came out from the original description of the human Nude/SCID phenotype and on the role of FOXN1 and of the other members of FOX subfamilies in those immunological disorders characterized by abnormal T-cell development or abnormal T-cell regulatory homeostasis.

Introduction: Severe Combined Immunodeficiencies

Primary immunodeficiency (PID) diseases are heritable disorders of immune system.[1] Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness. Apart from physical barriers, the immune response is composed from a diverse network of defenses, including cellular components and soluble mediators. A proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial

*Corresponding Author: Claudio Pignata—Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini, 5, 80131, Naples, Italy. Email: pignata@unina.it

Forkhead Transcription Factors: Vital Elements in Biology and Medicine, edited by Kenneth Maiese.

Table 1. Different genotypic forms of SCID classified on the basis of the immunological phenotype

Lymphocyte Phenotype	Form of SCID
$T^-B^+NK^-$	X-linked (deficit of γc)
	Deficit of Jak 3
	Deficit of CD45
$T^-B^+NK^+$	Deficit of IL-7Rα chain
	Deficit of CD3δ chain
$T^-B^-NK^-$	Deficit of Adenosine Deaminase
$T^-B^-NK^+$	Deficit of RAG1 or RAG2
	Deficit of Artemis
$T^{low}B^+NK^+$	Deficit of FOXN1

response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. The innate immune response involves three major cell types: phagocytic cells, such as neutrophils and macrophages, natural killer (NK) cells and antigen presenting cells, which are also involved in the induction of an adaptive immune response. The adaptive immune system includes T and B lymphocytes responsible for cellular and humoral responses, respectively. However, these components of immune system to maintain a normal resistance to infections act in a well orchestrated and integrated unique system.

In the last 5 decades, since the first human genetic defect was identified more than 200 PID syndromes have been described. PIDs can be divided into subgroups based on the component of the immune system that is predominantly affected, including T, B, NK lymphocytes, phagocytic cells and complement proteins (Table 1). The antibody deficiencies (B-cell or humoral immunodeficiencies) are characterized by a genetic lesion, that selectively affects antibody production, but a normal cell-mediated immunity. In the cellular deficiencies, cellular effector mechanisms are compromised, whereas antibody production is largely normal in that B-cell intrinsic machinery is intact. The combined immunodeficiencies are characterized by an impairment of both effector arms of the specific immunity, which results in a more severe clinical phenotype. However, since an efficient B-cell antibody response also depends on T-cell activation of B lymphocytes, defects in either cell type have the potential to affect both cellular and humoral immunity to varying degrees.

Of note, most of the diseases within the last category are due to genetically determined blocks in the T-lymphocyte differentiation program. In the absence of mature T-cells, adaptive immunity is abrogated, thus resulting in a broad-spectrum susceptibility to multiple pathogens also including opportunistic microorganisms. Overall, unrespectively of the pathogenic mechanism of the individual form of severe combined immunodeficiencies (SCIDs), a common hallmark of these diseases is the feature that bacterial, viral and fungal infections are often overwhelming.

The discovery of a so wide number of distinct clinical entities which differ in either the genetic cause or the altered immunological function led to an uncomparable increase in the knowledge of the intimate mechanism by which a proper immune response is generated. Intriguingly, most of the genes whose alterations underlie PID are selectively expressed in hematopoietic cells with a few exception as, for example, Ataxia Telangiectasia Mutated (ATM) gene, also expressed in Purkinje cells and Adenosine Deaminase (ADA) which is ubiquitous. This dogma, however, led to underestimate those novel immunodeficiencies, which have different features involving other nonhematopoietic tissues.

In 1996, a novel form of SCID (MIM 601705; Pignata guarino syndrome) was described and referred as the human equivalent of the well known murine phenotype named Nude/SCID.[2] This disease is the first example of SCID not primarily related to an hematopoietic cell abnormality, but rather to an intrinsic thymic epithelial cell defect .[3]

The Nude/SCID Phenotype

In 1966, S.P. Flanagan identified a new mouse phenotype that spontaneously appeared in the Virus Laboratory of Ruchill Hospital, Glasgow, UK, characterized by loss of the hair. This mouse showed an abnormal keratinization in hair fibers, with follicular infundibulum unable to enter the epidermis.[4] The mice also showed an inborn dysgenesis of the thymus[5] resulting in a compromised immune system lacking T-cells.

Subsequently, the molecular nature of the nude defect was characterized and attributed to a genetic alteration of the transcription factor FOXN1 (also called WHN or HFH11), mainly expressed in thymus and skin.[6-8] The analysis of the genomic sequence of the nude mouse revealed the presence of a single base pair deletion in exon 3, absent in the wild-type allele. This deletion led to a frameshift that resulted in an aberrant protein prematurely terminating in exon 6 and the loss of the postulated DNA binding domain.

The mouse nude mutation led to an abnormal development of the skin and thymus[4,9] and a severe alteration of the nails.[10] Later studies demonstrated that both defects, as lack of fur development and agenesis of the thymus, are pleiotropic effects of the same gene.[11] In particular, the skin of the nude mouse contains the same number of hair follicles as a wild-type control, but these follicles result in an uncomplete hair, that could not enter skin surface.[4,10] Flanagan analyzed carefully nude mouse skin and observed that at birth the hair follicles were normal, but by six days after birth the hair started to twist and coil, failing to penetrate the epidermis.[4] This hairless condition could be reverted by oral administration of cyclosporine A or recombinant keratinocyte growth factor (KGF or FGF-7), that influence the number of hair follicles or the cyclic hair growth.[12,13] Furthermore, the nude mouse epidermis shows failure in differentiation and a reduced number of tonofilaments are observed in spinous, granular and basal layers.[10] The nude *Foxn1* gene doesn't affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle.[14,15] In addition to these cutaneous abnormalities, nude animals develop an abnormal thymus, resulting in a severe T-cell deficiency and an overall severely impaired immune system. In fact, thymus morphogenesis is stopped at the first stages of development with no subcapsular, cortical and medullary regions formation, that characterizes a normal mature organ.[16] In addition, the observation that thymus restoration doesn't lead to hair growth demonstrated that the lack of the hair and the athymia were not related one to each other.[16,17]

Furthermore, the nude phenotype is characterized by nail malformations and poor fertility. The first condition is attributed to an abnormal production of filaggrin protein in nail matrix and nail plate, subsequent to a loss of keratin 1 protein. Differently, the second condition may be the result of changes in hormonal status, as demonstrated by the altered serum levels of estradiol, progesterone and thyroxine.[10]

For many years the human counterpart of nude mouse phenotype has been erroneously considered the DiGeorge syndrome, which occurs spontaneously and is mainly characterized by thymic hypoplasia or aplasia. However, several lines of evidence argue against the analogy between these two disorders. In fact, DiGeorge syndrome is often associated with neonatal tetany and major anomalies of great vessels. These defects are due to malformation of the parathyroid and heart, derived from a major embryologic defect in the third and fourth pharyngeal pouch from which the thymus primordium emerged. In addition, in this syndrome hairlessness is missing and gross abnormalities in skin annexa are not found. Children with DiGeorge syndrome also have lymphopenia, with a reduction of T-cells, that are poorly responsive to common mitogens.[18]

The discovery of the human phenotype completely equivalent to the nude mouse phenotype began with the identification of two sisters, whose clinical phenotype was characterized by congenital alopecia, eyebrows, eyelashes, nail dystrophy, as shown in Figure 1 and several T-cell immunodeficiencies, illustrated in Table 2.[2] The two patients were born from consanguineous parents who originated from a small community of South of Italy that may be considered geographically and genetically isolated, as below detailed. This led to consider the syndrome as inherited as an autosomal recessive disorder. The T-cell defect was characterized by a severe functional impairment, as shown by the lack of proliferative response to mitogens.

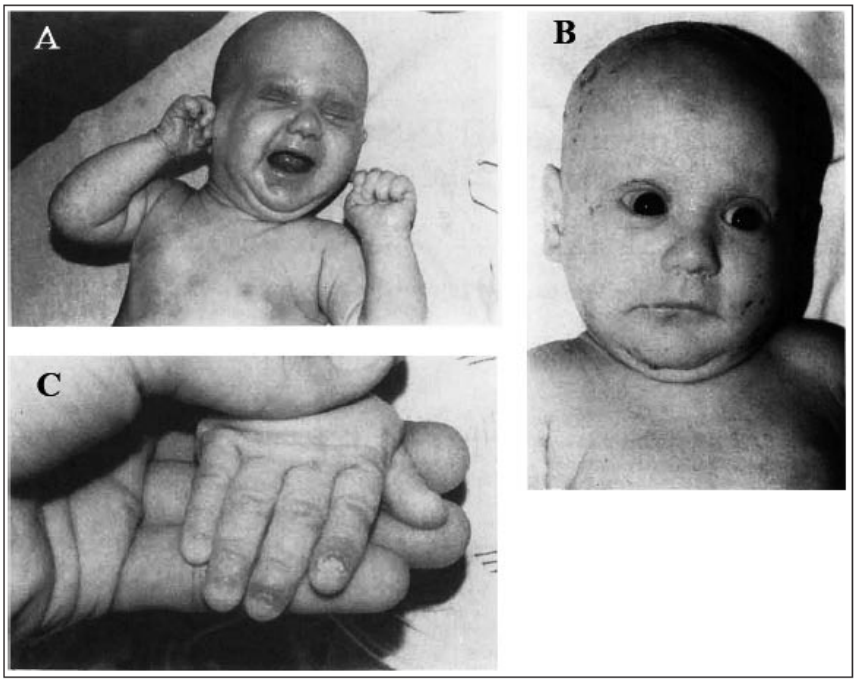


Figure 1. A,B) Alopecia of scalp, eyebrows and eyelashes in two sisters in whom the human Nude/SCID phenotype was first described. C) Nail dystrophy in human Nude/SCID. Reprinted with permission from: Pignata C, Fiore M, Guzzetta V et al. Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. Am J Med Genet 1996; 65:167-170.

Alopecia and nail dystrophy are also found in other syndromes, such as dyskeratosis congenita (DC).[19,20] However, this novel syndrome profoundly differed from DC, in that major signs, such as abnormal pigmentation of the skin and mucosal leucoplakia, were lacking in the Nude/SCID. Moreover, the immunological abnormalities were different from those reported in patients with DC in either the severity of clinical course or type of alterations.[21,22] Both Nude/SCID patients showed alopecia at birth and in one sib it still persisted after a bone marrow transplantation, thus ruling out that it was secondary to an acquired skin damage. This finding suggested that the alopecia in this patient was primitive in nature.[2] Furthermore, these features were similar to those reported in athymic mice, that completely lack body hair and in which restoration of a thymus did not lead to hair growth.[11] Taken together, these observations suggested that the association between alopecia and the immunodeficiency reported in the two sisters were linked to a single gene defect.[2]

Due to the similarities between the human clinical features and the mouse Nude/SCID phenotype, a molecular analysis of the *FOXN1* gene was performed in these patients and revealed the presence of a C-to-T shift at 792 nucleotide position in the cDNA sequence. This mutation leads to a nonsense mutation R255X in exon 5, with a complete absence of a functional protein[23] similar to the previously described rat and mouse *Foxn1* mutations.[24-26] In *humans*, *FOXN1* is located on chromosome 17[23] and encodes a transcription factor mainly expressed in the epithelial cells of the skin and thymus, where it maintains the balance between growth and differentiation.

Since the first description of these Nude/SCID patients, other patients with a similar phenotype were identified. In particular, a Nude/SCID patient was diagnosed in Portugal. The newborn presented with alopecia and nail dystrophy associated with severe infections. The screening for R255X mutation of *FOXN1* gene revealed that the patient was homozygous for the mutation. It should be noted that the patient was born to consanguineous parents, both from Lisbon (communicated to European Society for Immunodeficiencies, 2006).

Table 2. Major clinical and immunological features of the first identified human Nude/SCID patients. For more details see reference 3.

	Patient 1	Patient 2
Clinical features		
Alopecia	+	+
Nail dystrophy	+	+
Growth failure	+	+
Omen-like syndrome	+	–
Severe interstitial pneumopathy	?	+
Immunological features		
Percentage of positive cells		
T-cells (CD3)	32	25
B-cells (CD19)	63	37
NK cells (CD56)	23	25
Proliferative response to mitogens	Absent	Absent
Serum immunoglobulins		
IgG (g/L)	4.94	6.10
IgA (g/L)	0.49	0.43
IgM (g/L)	0.80	1.25
IgE (KU/L)	N.T.	2760
Specific antibody response		
Tetanus toxoid	Absent	Absent
Allohemoagglutinins	Absent	Low
HbsAg	Absent	Absent
WHN mutation	R255X	R255X

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In the village where the patients originated, additional patients of previous generations were affected with congenital alopecia and died early in childhood because of severe infections.[27]

A population study aimed to identify an ancestral founder effect for this phenotype was conducted in the village and in particular a genetic screening for the presence of the R255X mutation was performed. The study led to identify 55 subjects, corresponding to 6.52% of the studied population, who carried the mutation in heterozygosity.[27] The identification of the haplotype for the *FOXN1* locus, by analysing 47 chromosomes carrying the mutation R255X, led to identify the single ancestral event that underlies the human Nude/SCID phenotype. All the affected cases belonged to an extended seven-generational-pedigree, founded by a single ancestral couple born at the beginning of the 19th century from which four family groups originated. The pedigree analysis revealed that 33.3% of heterozygotes inherited the mutant allele from their mother, whereas 66.7% from their father.

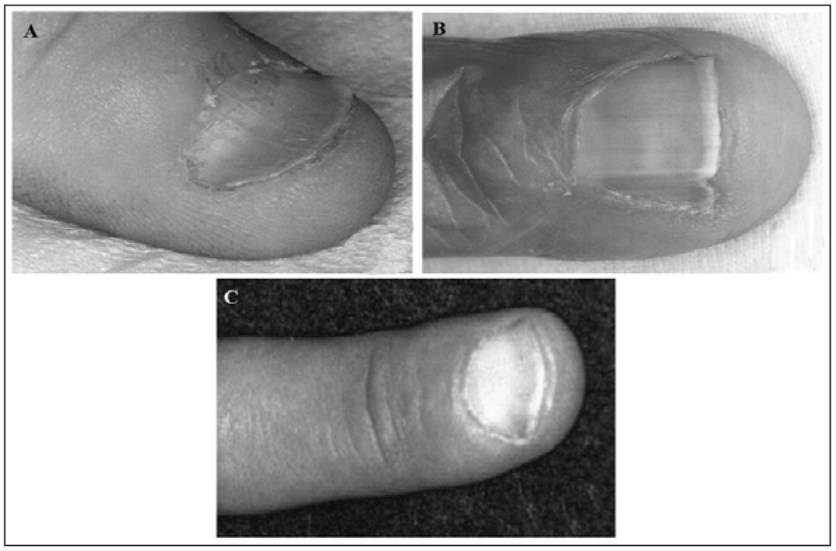

Figure 2. Nail dystrophy patterns in subjects heterozygous for the *FOXN1* mutation: A) koilonychia; B) canaliform dystrophy; and C) leukonychia. Reprinted with permission from: Auricchio L, Adriani M, Frank J et al. Nail distrophy associated with a heterozygous mutation of the Nude/SCID human *FOXN1* (*WHN*) gene. Arch Dermatol 2005; 141:647-648; ©2005 American Medical Association. All rights reserved.

Moreover, this pedigree was also characterized by a high rate of consanguineous matings, typical of a small community. In fact, 14 of 151 marriages were between consanguineous subjects.[27]

Subsequently, the identified heterozygous subjects were examined with a particular regard to ectodermal alterations, namely of hair and nails, in order to define whether the heterozygosity was associated with mild clinical signs. The examination revealed no association between gross alteration of the hair and heterozygosity, while 39 of the 55 heterozygous subjects showed a nail dystrophy.[28] Of note, this alteration was not found in other control subjects and was not related to an acquired form of nail dystrophy. The most frequent phenotypic alteration affecting the nails was koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself. Less frequently, a canaliform dystrophy and a transverse groove of the nail plate (Beau line) was also observed (Fig. 2).[28] However, the most specific phenotypic alteration was leukonychia, characterized by a typical arciform pattern resembled to a half moon and involving the proximal part of the nail plate. These alterations of digits and nails were also reported in a few strains of nude mice.[15] *FOXN1* is known to be selectively expressed in the nail matrix where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration.[28]

As this form of SCID is severe due to the absence of the thymus and the blockage of T-cell development, a screening program for prenatal diagnosis in this population was conducted for the identification of fetuses carrying the mutation. The genetic counselling offered to couples at risk led to identify two affected female fetuses during the first trimester of pregnancy, thus indicating the importance of this effort. Both fetuses were homozygous for the R255X mutation and the autoptical examination revealed the absence of the thymus and a grossly abnormal skin which was tighter than usual and which showed basal hyperplasia and dysmaturity, suggestive of an impaired differentiation program. Of note, one of the two fetuses also showed multiple-site neural tube defects, including anencephaly and spina bifida that could explain the high rate of mortality in utero observed in the described population. Intriguingly, the other forms of SCID become clinically evident in postnatal life, when the protection of the newborn by the mother immune system declines. In the community where the Italian patients originated, a high rate of prenatal mortality was observed. Moreover, there

was an evidence that the mouse *Foxn1* gene is also expressed in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain.[29] Even though no formal demonstration is available, a possible explanation for the prenatal mortality could be that *FOXN1* genetical alteration is also implicated in more severe development defects at least in the conditions of highest clinical expressivity. This could also explained the surprising long interval of time that elapsed between the description of mouse and human diseases.

Fox Family Members and Immune System

The forkhead box (FOX) gene family comprises a diverse group of 'winged helix' transcription factors that are involved in development, metabolism, cancer and aging. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions.[30] They were first recognized in *Drosophila*, but later they were also identified in other organisms, from *yeasts* to *humans*. The term FOX is now used to refer to all chordate forkhead transcription factors. A phylogenetic analysis led to classify all known FOX proteins in at least 15 subfamilies (named from A to Q)[31] on the basis of their structure; in each subfamily (or class), an individual gene is identified by a number. The crystal structure of the forkhead DNA binding domain is a 'winged helix' motif, consisting of three α helices flanked by two 'wings' of β strands and loops.[32] The structure and the amino acids sequence are highly conserved within species and family members.

The functional effect of all FOX proteins can be either the activation (transactivation) or the inhibition of gene transcription[33] in a wide range of context. *Fox* gene mutations can be associated with diverse phenotypes as cranio-pharyngeal developmental defect (*FOXE1*), speech and language abnormalities (*Foxp2*) and hearing loss (*Foxj1*).[34] Moreover, most of these winged helix proteins play crucial roles in several aspects of immune regulation. In particular, genetic alterations of at least four FOX family members, FOXP3, FOXJ1, members of the FOXO subfamily and FOXN1, result in paradigmatic immune disorders and well defined novel clinical entities.[33]

FOXP3 (scurfin, sf, JM2) is the most studied forkhead family member in immunology, because of its role in the pathogenesis of autoimmunity associated with immunological functional disorders.[35-37] FOXP3 was found to be expressed in $CD4^+$ $CD25^+$ regulatory T-cells (Treg), that represent a subset of $CD4^+$ T-cells bearing high levels of CD25 (the IL-2 receptor α-chain), whose role is to maintain self-tolerance by downregulating the reactivity of conventional $CD25^-$ $CD4^+$ helper T-cells.[35,38] *Foxp3* is also expressed in lymphoid organs, such as spleen and thymus, where it plays an essential role during development, allowing the differentiation of the Treg population. The study of scurfy mice (mice with X-linked recessive mutation in *Foxp3*) revealed an overproliferation of activated $CD4^+$ T-cells, resulting in dysregulation of lymphocyte activity.[39,40] The lack of DNA binding domain of the protein leads to death of hemizygous males at 16-25 days after birth[39,40] and in the surviving mice in a great exacerbation of the autoimmune phenotype.[41]

The corresponding human disorder is represented by Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX; also known as X-linked autoimmunity and allergic dysregulation syndrome, XLAAD). This fatal recessive disorder is due to truncated protein or inhibition of DNA binding domain. It develops in early childhood and is associated with protracted diarrhoea, thyroiditis, dermatitis, allergic manifestations, insulin-dependent-type 1 diabetes and anaemia, besides massive T-cell infiltration into the skin and gastrointestinal tract and high serum levels of autoantibodies, as a sign of autoreaction.

Recent studies have shown that the expression of FOXP3 and the subsequent conversion of human and mouse peripheral naïve T-cells in Treg is induced by Transforming Growth Factor-β (TGF-β).[42,43] Most probably, this event is mediated by activation of Small Mothers Against Decapentaplegic (SMAD) transcription factors. Generally, the inhibition of TGF-β-mediated signaling involves SMAD7 in an autoregulatory loop, but it was also shown that FOXP3 can inhibit it, as well. The induction of *FOXP3* expression by Treg results in a prolongation of TGF-β-mediated signaling, perhaps allowing the stabilization or expansion of the Treg pool.[33]

FOXOs are the mammalian homologues of the *Caenorhabditis elegans* dauer formation mutant 16 (DAF-16) and, in this organism, they seem to be involved in longevity regulation. FOXO1

(FKHR, forkhead in rhabdomyosarcoma), FOXO3A (FKHRL1, FKHR-like 1), FOXO4 (AFX, mixed lineage-leukemia (trithorax homolog) translocated to 7 homolog, Mllt7) and FOXO6[44] are the most studied members of this family for their implication in the regulation of apoptosis, cell cycle, metabolism and resistance to oxidative stress.[45-47] Gene targeting experiments in mice have demonstrated that FOXO1 regulates insulin sensitivity,[48,49] adipocyte differentiation[49] and angiogenesis,[50] while FOXO3A regulates ovarian development and fertility[51-53] and FOXO4 appears to be largely dispensable for gross organismal homeostasis.[52] FOXO proteins are ubiquitously expressed, even if there is a tissue specific expression for the diverse isoforms. While FOXO1 is ubiquitous, FOXO3A is expressed in lymphocytes and it appears the dominant isoform of the mammalian family. Cellular stimulation by mitogens or cellular stress, leads to activation of several intracellular kinases such as phosphatidyl inositol 3 kinase (PI3K), serum/glucocorticoid-regulated kinase (SGK) and protein kinase B (PKB, Akt), resulting in the phosphorylation of the FOXOs. This makes FOXO unable to bind DNA and renders it susceptible to 14-3-3-mediated nuclear export[54-59] and/or proteasome mediated degradation (IkB kinase (IKK))[60,61] thereby preventing FOXO-mediated transcription. In resting cells, unphosphorylated forms of the FOXOs are localized in the nucleus, where they are transcriptionally active and regulate several biologic processes, including proliferation, apoptosis and response to cellular stress.

To date, there is no evidence that an alterated FOXO activity is associated with a human immunological disease. However, in mice a significant diminished FOXO activity in T-cells is associated with autoimmune lupus syndrome, thus leading to hypothesize a possible relationship between the *FOXO* genes and inflammation in *humans*.[62] Differently, *FOXO* gene dysregulation has been well documented in human cancer.

The FOXJ1 (hepatocyte nuclear factor/forkhead homolog-4, HNF-4, FKHL-13) transcription factor plays an important role in the development of ciliated epithelia.[63-66] Thus, FOXJ1 is expressed in all structures containing ciliated cells, such as the lungs, spermatids, oviducts and choroid plexus.[67] The loss of FOXJ1 results in lethality in utero or soon after birth as demonstrated by observations of *Foxj1*$^{-/-}$ mice that die during embryonic development.[63,65] In fact, *Foxj1*-deficient mice are characterized by absence of cilia and, subsequently, suffer from significant developmental abnormalities including heterotaxy and hydrocephalus.[33] Besides its role in the differentiation of ciliated cells, recently a new role for FOXJ1 has been discovered in the differentiation of other cell types. It was observed that *Foxj1* is downregulated in lymphocytes isolated from mice affected with systemic lupus erythematosus (SLE); this evidence suggests that FOXJ1 might prevent autoimmune reactions.[68]

FOXJ1 is expressed in naïve T-cells and its downregulation occurs after interleukin-2 (IL-2) and/or T-cell receptor (TCR) stimulation.[68] FOXJ1, similarly to FOXO3A, is required in vivo to modulate NF-kB activity, upregulating IkBβ and maintains T-cell tolerance, but unlike FOXO3A deficiency, FOXJ1 deficiency is much more severe, affecting a different spectrum of organs and Th1 cytokine production. Thus, these two forkhead members play either overlapping or clearly distinct roles in helper T-cells, even though the intimate mechanisms remain to be elucidated.[69]

Foxn1, a highly conserved transcription factor, has been previously extensively mentioned. It exerts its function after activation through phosphorylation, that promotes its nuclear translocation.[54-56] Into the nuclei it interacts with DNA as a monomer through its forkhead box, but the target genes and the specific biochemical mechanism of interaction with the promoter regions remain to be elucidated.[32,70] FOXN1 expression is strongly regulated by wingless (Wnt) proteins[71] and bone morphogenetic proteins (BMPs)[72] in both autocrine and paracrine fashions[69] and its expression is restricted to epithelial cells in the skin[14] and in the thymus. There are no data available on FOXN1 mRNA expression in liver, spleen, testis, lung, heart and brain, but murine choroid plexus.[29] During embryogenesis, FOXN1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney and urinary tract. In adult life, its expression is limited to epithelial cells of the intestine, spermatocytes of the testis and thymus.[73] In particular, on the basis of the observation that nude mice keratinocytes do not differentiate in a normal fashion, FOXN1 could be considered as a key regulator of the balance between keratinocytes growth

and differentiation. It suppresses the involucrin and locrin expression, both components of the cornified envelope and the profilaggrin, involved in the aggregation of the intermediate filaments. Other factors have been identified as Foxn1 target. In fact, recent studies have shown that Foxn1 is linked to Akt (PKB) expression,[74] thus giving a possible explanation of FOXN1 involvement in epidermal layer regulation. FOXN1 also controls follicular formation, influencing the expression of two hair keratins, mHa3 and mHb5.[75]

Moreover, FOXN1 transcription factor regulates thymus epithelial cells differentiation. Null mutation of this protein led to an immature thymus, but the molecular mechanism used by FOXN1 in this context remains still unclear.

The human and mouse clinical phenotype associated with FOXN1 genetical alteration has been extensively described above.

FOXN1 Skin Specific Expression and T-Cell Development

So far, the thymic tissue has been considered the only organ with a unique capacity to support the generation of a functional population of human mature T-cells, thus expressing a diverse repertoire of antigen receptors.[76,77] In particular, within the mature and functional thymus, mature T-lymphocytes derive from the interaction between the thymic epithelial cells, that are the main component of the stroma and the T-cell precursors originated in the bone marrow.[78,79] Thymic epithelial cells are implicated in either thymus organogenesis or in most stages of maturation of thymocytes.[78,79] The absence of FOXN1, as in Nude/SCID phenotype, results, as previously extensively mentioned, in a thymus anlage that lacks the capacity to interact with the hematopoietic progenitor cells, thus precluding the maturation of thymocytes[80,81] and leading to the immunodeficiency.[16]

FOXN1 gene spans about 30 kilo bases (kb)[5] and it is composed of nine exons.[70] Interesting, an extensive screening of cDNA clones obtained from skin cells revealed the presence of two different first exons which are noncoding,[70] the exons 1a and 1b, that undergo to alternative splicing to either of two splice acceptor sites of the exon 2, located upstream of the initiation codon. This suggests the presence of two distinct promoters of exons 1a and 1b.[5] The alternative usage of the exon 1a or 1b seems to be tissue specific,[70] in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin.

In the interfollicular epidermis, FOXN1 expression parallels the onset of terminal differentiation. It is primarily expressed in the first suprabasal layer that contains keratinocytes in the early stages of differentiation, that have left the cell cycle and initiated terminal differentiation.[15] In the hair follicle, FOXN1 expression is restricted to a specific compartment, the supramatrical region,[15] where the cells stop to proliferate and begin terminal differentiation.[82] On the basis of these observations, FOXN1 could be considered a marker of transition from proliferation to a postmitotic state and an important regulator of the initiation of terminal differentiation.[5]

Of note, significant expression levels of FOXN1 were found in cultures containing skin cells along with hematopoietic precursor cells (HPCs), suggesting a role of human skin in supporting a full process of human T-cell development.[83] Although thymus and skin are different in their three-dimensional structure, experiments performed with keratinocytes and fibroblasts of the skin and HPCs obtained from bone marrow, reconfigured in a different three-dimensional arrangement, demonstrated the capacity of this "surrogate" organ to generate mature and functional T-cells from precursors.[83] Of note, these cells show the same characteristics of recent thymic emigrants such as the T-cell surface markers, including the CD3/TCR complex[84] and the TCR rearrangement excision circles (TRECs), derived from the recombination of TCR genes. These cells also possess a diverse TCR repertoire and can be considered mature and functional because they have full capacity to proliferate, express the activation antigen CD69 and produce cytokines in response to TCR/CD3 stimulation.[83] Thus, it is conceivable that skin and bone marrow derived cells can be potentially used to generate de novo mature, functional, diverse and self-tolerant T-cells. These data would imply their potential future therapeutic usage in patients with immunological disorders.[83]

The present chapter contains information of the recent works that came out from the original description of Nude/SCID phenotype. For the first time, only recently, a careful description of

clinical manifestations associated with an alteration of the *FOXN1* gene has been provided, thus leading to identify the human equivalent of the well studied spontaneous murine Nude/SCID immunodeficiency. In this context, alterations of FOXN1 and of other members of FOX subfamilies are now emerging as intriguing causes of immunological disorders mainly characterized by abnormal T-cell development or abnormal T-cell regulatory homeostasis.

Eventually, it should be underlined that the Nude/SCID phenotype is the only form of SCID associated with an alteration of a gene that is not expressed in the hematopoietic cell.

Novel knowledge in this field would be very helpful in the comprehension of the intimate mechanisms underlying T-cell ontogeny process in *humans* and in discovering novel clinical entities related to abnormalities of the process.

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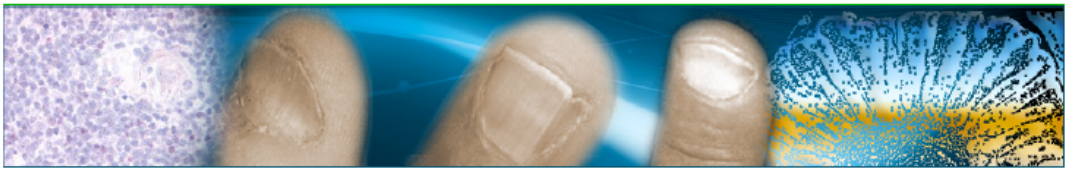


La sindrome Nude/SCID: dal modello murino al fenotipo umano

Claudio Pignata, Anna Fusco, Stefania Amorosi, Ilaria Vigliano, Valentina Genovese, Giuseppina Aloj e Leopoldo Valentino
Dipartimento di Pediatria, Università "Federico II", Napoli

Abstract

A proper normal immune response is initially based on the innate immunity, characterized by a rapid and nonspecific response to infections, and later on the adaptive immunity, characterized by a specific response to a particular pathogen. Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness.

Primary immunodeficiencies are congenital disorders of the immunological response, which can be divided into subgroups on the basis of the component of the immune system predominantly affected, including T, B, NK lymphocytes, phagocytic cells and complement proteins. The severe combined immunodeficiency (SCID), characterized by abnormalities of T, B and NK cells, consists of a group of distinct diseases associated with a severe clinical phenotype due to an impairment of both effector arms of the specific immunity.

In the 1996, a novel form of SCID (MIM 601705; Pignata guarino syndrome) was described, and proposed as the human equivalent of the well known murine phenotype described by Flanagan in 1966.

This murine model was defined as Nude/SCID. The hallmarks of the Nude/SCID phenotype are congenital alopecia, from which the term "Nude" for the spontaneous murine model, nail dystrophy and an intrinsic defect of the thymus, always associated with a profound T-cell defect.

The affected mice described by Flanagan, also showed an inborn dysgenesis of the thymus resulting in a compromised immune system lacking T cells. Moreover, molecular studies on the nude murine model led to identify Foxn1 as the gene responsible of the Nude phenotype. Also in humans as in mice, the molecular analysis reveals alterations in FOXN1 gene. Of note, the immunological phenotype of these patients is characterized by a marked reduction of CD3+, CD4+ and CD8+ cells and by the absence of naïve CD4+CD45RA+ cells.

It should be mentioned that studies performed in Nude/SCID mice gave a great contribution to the knowledge of cell-mediated immunity. In humans for a long time, the DiGeorge syndrome (DGS) was erroneously considered the human counterpart of the murine Nude/SCID phenotype. However, because of the profound differences among DGS and mouse Nude/SCID, the mouse model has been considered misleading to understand T-cell ontogeny in humans.

The description of the human equivalent of the Nude/SCID syndrome unravelled many of the dilemmas of T-cell ontogeny in man.

Novel knowledge in this field would be very helpful in the comprehension of the intimate mechanisms underlying the T-cell differentiation process in humans and in discovering novel clinical entities related to abnormalities of the process.

1. **Le immunodeficienze gravi combinate**

Le immunodeficienze primitive rappresentano un ampio gruppo di disordini ereditari in cui la funzionalità del sistema immune risulta alterata (1). Normalmente, una risposta immune appropriata si avvale, inizialmente, dei meccanismi della risposta immunitaria innata che interviene rapidamente e in maniera aspecifica contro le infezioni ed, in seguito, di una risposta adattativa in grado di rispondere in maniera specifica contro un determinato patogeno. La risposta immune innata coinvolge principalmente tre tipi cellulari: le cellule fagocitiche, quali neutrofili e macrofagi, le cellule Natural Killer (NK) e le cellule presentanti l'antigene che, peraltro, sono coinvolte anche nell'induzione della risposta immune adattativa. Il sistema dell'immunità adattativa include i linfociti T e B che sono responsabili della risposta cellulo-mediata o umorale, rispettivamente. In ogni caso, per garantire la normale funzione del sistema immune e un'appropriata difesa dalle infezioni, tutte le diverse componenti devono lavorare in un sistema unico ben orchestrato.

Nelle ultime 5 decadi dall'identificazione del primo difetto immunitario umano su base genetica, sono state descritte più di 200 sindromi da immunodeficienza primaria (PID), la cui caratterizzazione ha permesso di acquisire nuove conoscenze sull'intimo meccanismo d'azione di un'appropriata risposta immune.

Delle PID fanno parte le immunodeficienze gravi combinate (SCID), ovvero disordini geneticamente determinati che possono compromettere sia la risposta cellulo-mediata, che quella umorale. Le SCID, infatti, sono caratterizzate da un'alterata funzionalità delle cellule T, B ed NK, che determina una maggiore suscettibilità a contrarre infezioni gravi che possono risultare fatali nei primi mesi di vita se non trattate correttamente.

Ad oggi, sono state descritte più di 7 forme diverse di SCID associate ad un difetto genetico noto e, sulla base del fenotipo immunologico associato a queste alterazioni molecolari, è stata proposta, e attualmente accettata con consenso unanime, una classificazione di questi disordini che si basa sulla presenza o meno di ciascuna delle 3 popolazioni maggiori.

Fenotipo linfocitario	*Tipo di SCID*
$T^-B^+NK^+$	X-linked (alterazione di γc) Alterazione di Jak 3 Alterazione di CD45
$T^+B^+NK^+$	Alterazione della catena alfa di IL-7R Alterazione della catena delta di CD3
$T^-B^-NK^-$	Deficit di Adenosina Deaminasi
$T^-B^-NK^+$	Alterazione di RAG1 o RAG2 Alterazione di Artemis
$T^{low}B^+NK^+$	Alterazione di FOXN1

Tabella 1. Classificazione delle forme di SCID con difetto genetico noto sulla base del fenotipo linfocitario.

La maggior parte delle SCID è causata da mutazioni di geni selettivamente espressi nelle cellule ematopoietiche, ad eccezione del gene dell'Adenosina Deaminasi (ADA) la cui espressione è ubiquitaria. Ciò giustifica il fatto che nella maggior parte delle SCID sia affetto solo il sistema ematopoietico e che, quindi, le infezioni resistenti al trattamento siano il principale campanello di allarme per le immunodeficienze. In realtà, considerare le infezioni come unico campanello d'allarme per la diagnosi di immunodeficienza ha portato a sottostimare quelle nuove immunodeficienze, che si presentano con caratteristiche differenti e che coinvolgono altri tessuti non di origine ematopoietica.

Un esempio è rappresentato da una nuova forma di SCID descritta, per la prima volta, nel 1996 (MIM 601705; Pignata Guarino syndrome) (2). Si tratta dell'equivalente umano del ben noto fenotipo murino definito come Nude/SCID e rappresenta il primo esempio di SCID non primariamente correlata ad un'anomalia delle cellule ematopoietiche, ma piuttosto ad aplasia timica (3). Uno dei segni peculiari del fenotipo Nude/SCID è l'alopecia congenita, da cui deriva il termine "Nude" usato per l'equivalente murino descritto da Flanagan nel 1966 (4), che è sempre associata ad un grave difetto del compartimento dei linfociti T.

2. **La sindrome nude/scid**

Dal fenotipo murino…

Nel 1966, Flanagan identificò un nuovo fenotipo spontaneo di topo caratterizzato da disgenesia congenita del timo (5) e da perdita del manto pilifero (4). Negli anni successivi, lo studio più attento di questo nuovo modello murino ha portato all'identificazione del gene Foxn1 quale fattore implicato nella patogenesi di entrambi i difetti analogamente a quanto descritto nel ratto (6). Foxn1 codifica, infatti, per un fattore di trascrizione (anche noto come Whn o Hfh11) espresso selettivamente nel timo e nella pelle, dove è coinvolto per lo più nei processi di differenziamento terminale delle cellule epiteliali (7-9).

L'analisi della pelle del topo Nude ha rivelato un numero normale di follicoli piliferi,

che, però, rispetto al topo wild-type, dopo 6 giorni dalla nascita, s'intrecciano inducendo uno sviluppo incompleto del pelo incapace di penetrare nello strato superficiale della pelle (4, 10). Quest'ultima caratteristica è il risultato di un'alterazione dell'omeostasi tra crescita e differenziazione dei cheratinociti nel follicolo pilifero (11, 12), che provoca, inoltre, alterazioni della cheratina 1 nella matrice e nella lamina ungueale determinando malformazioni delle unghie.

Accanto alle anomalie cutanee, tuttavia, il difetto principale dei topi Nude riguarda il sistema immunitario. In particolare, nei topi Nude la morfogenesi del timo è bloccata ai primi stadi di sviluppo con mancata formazione delle regioni sottocapsulare, corticale e midollare, che caratterizzano un normale organo maturo (13).

La mancanza del timo determina di conseguenza l'assenza di tutta la popolazione cellulare di derivazione timica nonostante il numero di precursori delle cellule T sia normale. La perdita di tutto il compartimento T determina una grave immunodeficienza come dimostrato dall'incapacità di sviluppare un aumento della cellularità a livello linfonodale in seguito ad iniezione locale di fitoemoagglutinina (PHA) e da ridotti livelli di immunoglobuline (14). Tali caratteristiche deteminano una totale compromissione della funzionalità del sistema immune.

Va sottolineato che nei 40 anni intercorsi dall'originaria descrizione del topo Nude sono stati pubblicati oltre 30000 lavori immunologici su riviste ad alto impact factor, che hanno permesso di elucidare i principali meccanismi dell'immunità cellulo-mediata. In buona sostanza, una considerevole parte del corpus doctrinae sull'immunità ritardata è stata acquisita grazie alla scoperta del topo Nude.

A causa dell'agenesia congenita del timo, i topi Nude sono stati considerati a lungo il modello animale di riferimento per la sindrome di DiGeorge, caratterizzata da ipoplasia o aplasia timica.

La sindrome di DiGeorge è caratterizzata da linfopenia, ridotto numero di cellule T e bassa risposta ai comuni mitogeni (14). Spesso, tale sindrome si associa a tetania neonatale e ad anomalie dei grandi vasi dovute a malformazioni delle paratiroidi e del cuore, che derivano da un difetto embrionale della terza e quarta tasca faringea da cui ha origine il timo primordiale. Tuttavia, i pazienti affetti presentano una minore compromissione della funzionalità immunitaria. Va segnalato che le gravi anomalie della risposta cellulo-mediata presenti nel topo Nude non si riscontrano nella sindrome di DiGeorge.

La scoperta del fenotipo umano Nude/SCID nel 1996, con la conferma delle anomalie immunologiche presenti nel topo Nude, ha definitivamente chiarito che le sindromi Nude/SCID e DiGeorge rappresentano 2 entità completamente distinte sotto il profilo patogenetico.

... al fenotipo umano

Nel 1996, dopo oltre 30 anni dall'identificazione del modello murino spontaneo, è stato descritto l'equivalente umano del fenotipo Nude/SCID (2). La scoperta della controparte umana del fenotipo murino è iniziata con l'identificazione di due sorelle, che presentavano un fenotipo clinico, ereditato come disordine autosomico recessivo, caratterizzato da alopecia congenita, estesa a ciglia e sopracciglia, e distrofia ungueale associate ad una grave immunodeficienza combinata con predominante compromissione delle cellule T (2).

Il difetto a carico delle cellule T comportava una grave alterazione funzionale, come dimostrato dalla perdita della risposta proliferativa nelle due pazienti. Queste caratteristiche cliniche erano simili a quelle riportate nei topi atimici (15) e, inoltre, il meccanismo molecolare che determinava la malattia dell'uomo era identico a quello descritto nel topo e nel ratto (16, 17).

Infatti, la malattia è dovuta ad un'alterazione del gene FOXN1, situato sul cromosoma 17 (18), che anche nell'uomo codifica per il fattore di trascrizione "winged-helix" espresso selettivamente nelle cellule epiteliali della pelle e del timo, dove regola l'equilibrio tra crescita e differenziazione. La mutazione più frequente riscontrata è stata finora la sostituzione C792T nella sequenza del cDNA di FOXN1, che determina la sostituzione R255X nell'esone 5, responsabile dell'assenza completa della proteina (18).

Dopo la prima descrizione di questo fenotipo nell'uomo, sono stati identificati anche altri pazienti con un fenotipo simile. In particolare, è stato identificato un paziente di origini afro-francesi che, a differenza degli altri pazienti Nude/SCID, presentava la mutazione R320W nell'esone 6 del gene FOXN1.

Un altro paziente, invece, di origine portoghese presentava alopecia e distrofia ungueale associate a gravi infezioni. L'analisi molecolare del gene FOXN1 rivelava la presenza della mutazione R255X in omozigosi.

E' interessante notare che il paziente portoghese era nato da genitori consanguinei, analogamente alle 2 sorelle in cui è stato descritto il fenotipo per la prima volta.

Le caratteristiche cliniche che accomunano tutti i pazienti Nude/SCID sinora descritti sono la comparsa di eritrodermia, la diarrea intrattabile, difetti di crescita e una storia di gravi infezioni recidivanti resistenti al trattamento con insorgenza nei primi 6 mesi di vita.

Segni	*Fenotipo Nude/SCID (Assenza di FOXN1)*
Clinici	
Atimia	Costante
Alopecia	Costante
Distrofia ungueale	Costante
Difetto di crescita	Frequente
Eritroderma	Frequente
Infezioni gravi	Costanti
Età d'esordio della prima infezione	< 6 mesi
Anomalie dello sviluppo neuronale	Incerte
Immunologici	
Conta assoluta dei linfociti	Normale
Linfociti $CD3^+$	Marcatamente ridotti (range: 0 – 25 %)
Linfociti $CD3^+CD4^+$	Assenti/marcatamente ridotti (range: 0 – 20 %)
Linfociti $CD3^+CD8^+$	Ridotti (range: 0 – 11 %)
Linfociti $CD16^+CD56^+$	Normali
Linfociti $CD19^+$	Normali/elevati (range: 37 – 75 %)
Linfociti T naïve	Estremamente ridotti (range: 0 – 3,1 %)
Risposta proliferativa ai mitogeni	Assente
Produzione di anticorpi specifici	Assente/molto compromessa
Livelli sierici delle immunoglobuline	Normali/ridotti

Tabella 2. Principali segni clinici ed immunologici associati al fenotipo umano Nude/SCID

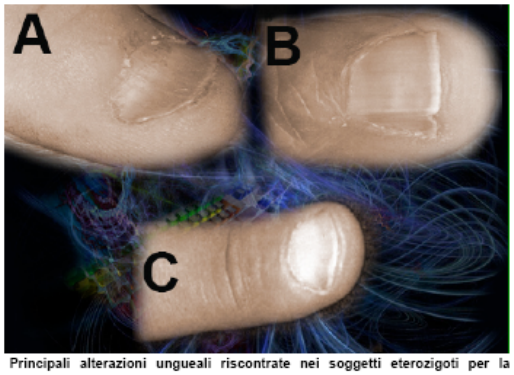
Dal punto di vista immunologico, i pazienti mostrano un difetto T selettivo testimoniato dall'assenza di risposta proliferativa associata ad un grave blocco nel differenziamento delle cellule T (2). In particolare, il fenotipo immunologico è caratterizzato da una drastica riduzione delle cellule CD3+, CD4+, CD8+ e dall'assenza di cellule naive CD4+CD45RA+. E' interessante notare che in tutti i pazienti descritti i linfociti B ed NK sono in numero normale.

Inoltre tutti mostravano alopecia alla nascita che, nei pazienti trattati con terapia con cellule staminali, persisteva anche dopo il trapianto, così da escludere che essa potesse essere secondaria ad un danno acquisito della pelle. Queste caratteristiche cliniche erano quindi in sintesi simili a quelle riportate nei topi atimici (15).

Da uno studio di popolazione eseguito nel paese di origine dei primi pazienti descritti, venivano identificati altri pazienti, appartenenti alle generazioni precedenti, affetti da alopecia congenita e morti nella prima infanzia a causa di gravi infezioni (19). Tali studi hanno inoltre permesso di identificare un effetto fondatore ancestrale, responsabile del fenotipo Nude/SCID in questa popolazione, che può essere considerata isolata sia per la posizione geografica del paese che sotto il profilo genetico. Lo studio di popolazione ha permesso, altresì, di identificare 55 soggetti eterozigoti per la mutazione R255X, che corrispondono al 6.52% della popolazione studiata (19). Tutti i soggetti affetti appartenevano ad un esteso pedigree di 7 generazioni, originato da una singola coppia ancestrale nata all'inizio del XIX secolo, da cui discendevano quattro gruppi di famiglie. L'analisi del pedigree rivelava, inoltre, la presenza di un alto tasso di matrimoni tra consanguinei (14 su 151), tipici delle piccole comunità (19). La conferma del singolo evento ancestrale alla base del fenotipo umano Nude/SCID è venuta anche dall'identificazione dell'aplotipo associato al locus FOXN1, ottenuto dall'analisi di 47 cromosomi portatori della mutazione R255X (19).

I soggetti eterozigoti identificati sono stati studiati con particolare attenzione per le alterazioni riscontrate a livello dei peli e delle unghie, al fine di definire un'eventuale associazione della mutazione in eterozigosi con segni clinici più lievi. L'analisi non ha rivelato alcuna associazione tra le alterazioni dei peli e lo stato di eterozigosità della mutazione, mentre, la distrofia ungueale è stata riscontrata in 39 soggetti sul totale dei 55 eterozigoti (20).

Va sottolineato, inoltre, che questa alterazione non veniva riscontrata nei soggetti controllo e non era correlata a nessuna forma acquisita di distrofia ungueale. La più frequente alterazione fenotipica riscontrata nelle unghie era la coilonichia ("unghia a cucchiaio"), caratterizzata da una superficie concava e dalle estremità del letto ungueale rialzate, associate ad un notevole assottigliamento del letto ungueale stesso. Le alterazioni meno frequenti, invece, erano la distrofia canaliforme e la scanalatura trasversa delle unghie (Beau line) (20). Le principali alterazioni ungueali descritte sono illustrate in figura.



Principali alterazioni ungueali riscontrate nei soggetti eterozigoti per la mutazione R255X del gene FOXN1. A) Coilonichia; B) Distrofia canaliforme; C) Leuconichia.

Tra tutte, l'alterazione fenotipica più specifica era la leuconichia, caratterizzata da un assetto tipicamente arciforme somigliante ad una mezza luna che coinvolgeva la parte prossimale del letto ungueale. Alterazioni delle dita e delle unghie erano state anche riportate in alcuni topi Nude (15).

Questi studi hanno consentito di offrire a questa popolazione un programma di screening per la diagnosi prenatale di questa grave forma di SCID. La consulenza genetica offerta alle coppie a rischio mediante la diagnosi prenatale effettuata con l'analisi diretta del gene FOXN1, ha portato sinora all'identificazione, durante il primo trimestre di gravidanza, di 2 feti affetti, dimostrando così l'importanza di tale screening. Entrambi i feti erano omozigoti per la mutazione R255X e l'esame autoptico ha rivelato, in entrambi, l'assenza del timo e anomalie della pelle che appariva più sottile del normale e che mostrava iperplasia basale e dismaturità, suggestive di un alterato programma di differenziazione. Inoltre, uno dei due feti identificati mostrava anche difetti multipli del tubo neurale, tra cui anencefalia e spina bifida.

Tali alterazioni potrebbero spiegare l'alto tasso di mortalità in utero osservato nella popolazione descritta non giustificabile con la SCID, che diventa clinicamente evidente solo dopo la nascita, quando al neonato inizia a mancare la protezione da parte del sistema immune della madre. Tali osservazioni fanno ipotizzare che la mutazione responsabile del fenotipo Nude/SCID e l'anencefalia possano essere casualmente correlate. A supporto dell'ipotesi di una diretta implicazione del gene FOXN1 nello sviluppo di difetti del tubo neurale, ci sono evidenze che il gene Foxn1 murino è espresso anche nelle cellule epiteliali del plesso coroideo, una struttura che riempie il terzo e il quarto ventricolo laterale del cervello embrionale (21). Pertanto, queste osservazioni suggeriscono un possibile ruolo di FOXN1 anche come cofattore nello sviluppo dei sistemi vitali fondamentali per un corretto sviluppo del feto.

Infine, va sottolineato che il fenotipo Nude/SCID umano è stato descritto nelle sue manifestazioni cliniche soltanto di recente. L'identificazione di nuovi casi ed ulteriori studi patogenetici potrebbero essere di notevole aiuto, analogamente al modello murino, nella comprensione degli intimi meccanismi dell'ontogenesi delle cellule T nell'uomo ancora non completamente elucidati.

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Brain alteration in a Nude/SCID fetus carrying *FOXN1* homozygous mutation

Stefania Amorosi [a,1], Ilaria Vigliano [a,1], Ennio Del Giudice [a], Luigi Panico [b], Giuseppe M. Maruotti [c], Anna Fusco [a], Mario Quarantelli [d], Carla Ciccone [b], Matilde V. Ursini [e], Pasquale Martinelli [b], Claudio Pignata [a,*]

[a] Department of Pediatrics, "Federico II" University, Naples, Italy
[b] Unit of Pathology, National Relevance Hospital "S.G. Moscati", Avellino, Italy
[c] Department of Gynecology and Obstetrics, "Federico II" University, Naples, Italy
[d] Biostructure and Bioimaging Institute, CNR, Naples, Italy
[e] Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Naples, Italy

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ABSTRACT

A critical role of the *FOX* transcription factors in the development of different tissues has been shown. Among these genes, *FOXN1* encodes a protein whose alteration is responsible for the Nude/SCID phenotype. Recently, our group reported on a human Nude/SCID fetus, which also had severe neural tube defects, namely anencephaly and spina bifida. This led to hypothesize that *FOXN1* could have a role in the early stages of central nervous system development. Here we report on a second fetus that carried the R255X homozygous mutation in *FOXN1* that has been examined for the presence of CNS developmental anomalies. At 16 postmenstrual weeks of gestation, the abdominal ultrasonography of the Nude/SCID fetus revealed a morphologically normal brain, but with absence of cavum septi pellucidi (CSP). Moreover, after confirmation of the diagnosis of severe Nude/SCID, the fetus was further examined postmortem and a first gross examination revealed an enlargement of the interhemispheric fissure. Subsequently, a magnetic resonance imaging failed to identify the corpus callosum in any section. In conclusion, our observations did not reveal any gross abnormalities in the CNS anatomy of the Nude/SCID fetus, but alteration of the corpus callosum, suggesting that *FOXN1* alterations could play a role as a cofactor in CNS development in a similar fashion to other *FOX* family members.

1. Introduction

A critical role of the Forkhead box (*FOX*) transcription factors in the development of different tissues has been shown in a number of studies where *FOX* genes have been inactivated by gene targeting or mutations [1]. In particular, among these genes, *FOXN1* encodes a protein selectively expressed in the epithelial cells of the skin and thymus of mice, rats and humans [2]. Its alteration is responsible for the Nude/SCID phenotype, characterized by congenital alopecia, nail dystrophy and severe combined immunodeficiency associated with a profound T-cell defect [3].

Recently, our group reported on a human fetus exhibiting the Nude/SCID phenotype due to *FOXN1* gene mutation which also showed severe neural tube defects, namely anencephaly and spina bifida. The affected fetus was identified during a prenatal genetic counselling program offered to at-risk couples in a Southern Italian village where a high frequency for mutated *FOXN1* has been documented. This led to hypothesize that *FOXN1* could have a role in the early stages of central nervous system (CNS) development [4], as shown for other *FOX* family members.

2. Case report

We report on a second fetus belonging to the same family, who carried the R255X homozygous mutation in *FOXN1*, examined for the presence of CNS developmental anomalies. Prenatal diagnosis, performed by villocentesis at 11 postmenstrual weeks of gestation, led to a diagnosis of Nude/SCID syndrome. At 16 postmenstrual weeks of gestation, the abdominal sonography, performed through GE Voluson E8 Ultrasound Machine, of the Nude/SCID fetus revealed a morphologically normal brain, apart from a probable absence of the septum pellucidum, as suggested by failure to visualize cavum septi pellucidi (CSP) by means of ultrasound, in that only the medial wall of the frontal horn could be detected (Fig. 1a). At this gestational age, CSP is a fluid-filled cavity located in between the two layers of the septum pellucidum. It is bounded superiorly and anteriorly by the corpus callosum and inferiorly by the fornix and is considered as a marker of a normally developed brain [5]. This structure (Fig. 1b) is seen in the 40% of cases at 15 weeks, 82% at 16–17 weeks and 100%

* Corresponding author. "Federico II" University, Naples, via Pansini 5, Italy. Tel.: +39 081 7464340; fax: +39 081 5451278.
E-mail address: pignata@unina.it (C. Pignata).
[1] Contributed equally to this manuscript.

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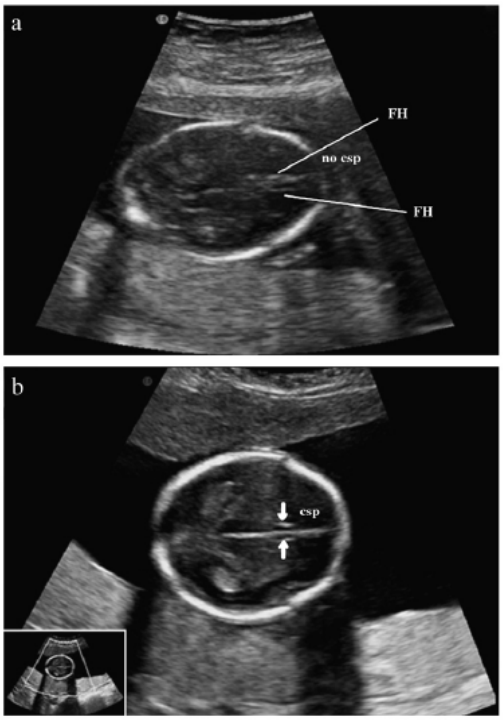

Fig. 1. (a) abdominal sonography does not reveal any structure on the cerebral midline to refer as CSP. Only medial wall of the frontal horn (FH) is evident. (b) sonography of a normal fetus at 16 weeks of gestation. The arrows indicate CSP.

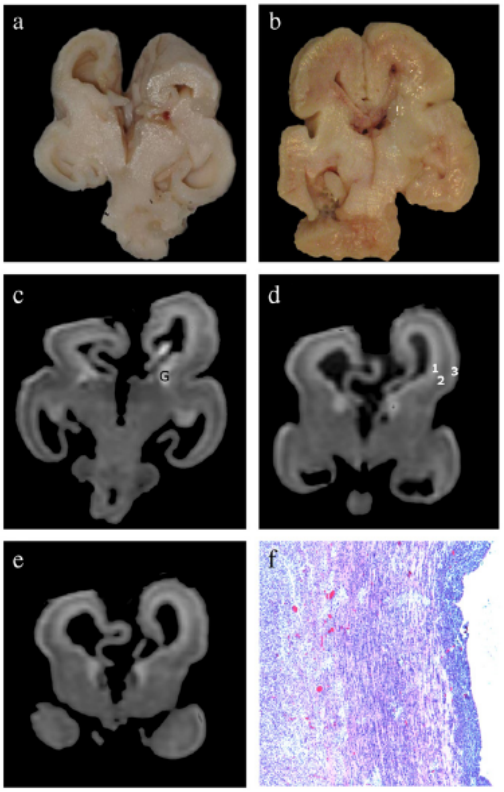

Fig. 2. (a) a coronal section of the brain, crossing the brainstem and the midbrain, in a Nude/SCID fetus revealed the abnormality in the development of corpus callosum. (b) a coronal section of the brain, in a control fetus of the same gestational age, revealed well formed midline structures with the presence of the corpus callosum, whose fibers cross the midline and of the septum pellucidum. (c–e) MRI showing the integrity of the major structures. Corpus callosum could not be identified in the three parallel coronal planes at the level of the brainstem (c) mammillary bodies (d) and nucleus accumbens (e). Ganglionic eminence (G) is labelled and transient fetal layers, including periventricular germinal matrix and intermediate zone (1), subplate zone (2) and cortical plate (3) are indicated. (f) Brain section stained showing the presence of Probst bundles, located medial to the lateral ventricle. H and E stain, X 50.

after the 18th week of gestation [5]. In agreement with parents' will, the pregnancy was terminated at 18th week and the fetus was further examined post mortem once obtained informed consent from the parents. At autopsy, the brain weight was 35 g and a first gross examination revealed an enlargement of the interhemispheric fissure.

In the Nude/SCID fetus, autopsy failed to reveal any macroscopic abnormality of brain structures, including choroid plexus, except the abnormality in the development of corpus callosum (Fig. 2a). Moreover, a coronal section of the brain, crossing the brainstem and the midbrain, in a control fetus of the same gestational age, revealed well formed midline structures with the presence of the corpus callosum, whose fibers cross the midline, and of the septum pellucidum (Fig. 2b). MRI study was performed on autopsy paraformaldheyde fixed samples of brain at 3 Tesla (Magnetom TRIO, Siemens, Germany) using a 3D low-angle, gradient-echo sequence (TR/TE 572/3.7 msec, FA 9°, FOV150mm, acquisition matrix 144×256, slice thickness 600 micrometers, using a quadrature volumetric coil) providing a T1w volume which was resliced along axial, coronal and sagittal planes. Images were processed using GIMP (http://www.gimp.org) to remove paraformaldehyde background. Brain MRI showed the integrity of major structures, but some mechanical damages due to extraction procedures. Cerebellum, brainstem, midbrain and thalami were normally formed and ganglionic eminence could be clearly defined. External morphology of cerebral hemispheres as well as the lamination pattern of the fetal telencephalic wall were normal for gestational age [6,7]. On the contrary, corpus callosum could not be identified in any section (Fig. 2c–e). Furthermore, in keeping with this, subsequent histological examination of a coronal section of the brain revealed the presence of longitudinally oriented bundles of white matter, formed by arrested axons that do not cross the midline (Probst bundles), located medial to the lateral ventricle (Fig. 2f).

3. Discussion

In conclusion, the present study did not reveal any gross abnormalities in the CNS anatomy of the Nude/SCID fetus. As compared to our previous observation this would imply that *FOXN1* alterations are not sufficient to induce neurulation anomalies [4]. However, the presence of a developmental anomaly of the corpus callosum would suggest that *FOXN1* alterations could play a role as a cofactor in CNS development in a similar fashion to other *FOX* family members, such as FoxP1, that helps Hox proteins to regulate the genes that control motor-neuron diversification [8,9]. It should be noted that the zebrafish orthologue of the mouse nude gene *Foxn1* is expressed in the developing eye and several other brain structures [10]. In

addition, we previously found that *FOXN1* gene is expressed in the choroid plexus of mice during CNS development [4]. Recently, it has also been shown that *Foxn1* is required to maintain the expression in the hair follicle matrix of Notch1 [11], where Notch1 signaling is known to regulate cell fate specification and pattern formation in the developing nervous system [12]. Nevertheless, the precise role of the *FOXN1* transcription factor in CNS development remains to be fully clarified.

Our case should encourage physicians and pathologists to search for *FOXN1* alterations in congenital brain developmental abnormalities.

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§ 1.2 T-cell ontogeny in the absence of the thymus: a comparative study between Nude/SCID and DiGeorge syndromes.

In nude mice the thymic agenesis is congenital and it closely resembles thymic agenesis of newborns affected with DiGeorge syndrome (DGS). DiGeorge syndrome represents the most-frequent microdeletion syndrome in humans, with an estimated incidence of 1 in 4000 live births [51-53]. It is classified as a 22q11 microdeletion, along with velo-cardio-facial syndrome and conotruncal anomaly face syndrome. DGS is clinically heterogeneous because of the loss of different genes in the region 22q11 [54, 55]. In particular, approximately 90% of the patients share a common 3 Mb hemizygous deletion of 22q11.2, encompassing approximately 30 genes and 8% of the patients have smaller deletions of 1.5 Mb encompassing 24 genes [54]. A few DGS cases have defects in other chromosome, notably 10p13 [56, 57]. 22q11.2 deletion syndrome is also associated with neonatal hypocalcaemia due to hypoplasia of the parathyroid glands and susceptibility to infection due to thymic hypoplasia occurs in up to 80% [58, 59]. A variety of cardiac malformations may be seen including tetralogy of fallot and interrupted aortic arch [60]. Patients may exhibit dysmorphic features with micrognathia and auricular abnormalities (including low set ears and abnormal pinna folding). Dysmorphic facial appearance in an individual with cardiac abnormalities or history of recurrent infection should raise suspicion for the diagnosis of 22q11.2 deletion syndrome [61]. A lot of studies have also reported an increased incidence of learning difficulties, behavioural problems and psychiatric disorders, [62] but the molecular origin of these neurobehavioral defects remains to be elucidated.

A variety of mouse models phenocopying DiGeorge syndrome have shed new light on crucial genes involved in the aetiology of the DiGeorge syndrome [54, 63]. They have also revealed the role of genes that are not present in the microdeletion, but which modulate the severity and penetrance

of the DiGeorge-like phenotype [55]. All gene mutations described so far have been shown to affect the development of the pharyngeal apparatus, a vertebrate-specific, transient embryonic structure [55]. DiGeorge syndrome is a congenital anomaly characterized by defects derived mainly from the third and fourth pharyngeal pouches, with additional abnormalities possibly extending from the first to sixth pharyngeal arch and first to fifth pharyngeal pouch [64]. Typically, the heart, the parathyroids, and the thymus are involved [65, 66]. Formation and patterning of the pharyngeal apparatus involves organization and interaction of different cell types derived from all embryonic tissue layers [55]. The pharyngeal system consists of five pharyngeal arches appearing in the lateral wall of the foregut between 8 and 11 days of embryonic development in the mouse and between 2 and 7 weeks of gestation in humans. The arches are separated from each other externally by ectoderm-lined pharyngeal clefts and internally by endoderm-lined pharyngeal pouches. The five arches develop in anterior-to-posterior order, and are numbered from 1 to 6 (the fifth arch, which is buried, is termed the sixth arch by convention). Morphogenesis of the pharyngeal apparatus involves the initial segmentation and expansion of pharyngeal arches and pouches, followed by development of pharyngeal derivatives from these structures. Genetic pathways regulate these processes in a complex spatial and temporal manner, but the players and molecular interactions involved in these dynamic genetic networks are only partially understood [55]. In 22q11.2 deletion syndrome the defects in the thymus, parathyroid and conotruncal regions of the heart are believed be caused by impaired migration of neural crest cells into pouch ectoderm. Disruption in the pathways of neural crest cell development in mice results in malformations similar to the 22q11.2 deletion phenotype.

A variety of candidate genes have been identified [61]. Engineered segmental deletions of the murine Df1 regions syntenic to the human

22q11.2, transgenic complementation studies of genes within this locus, and single-gene-targeting experiments have revealed the significance of the transcription factor Tbx1 for the development of the pharyngeal apparatus and its derivates {Lindsay, 1999 #1781;Lindsay, 2001 #1918;Merscher, 2001 #1919; [26]. Mice with these genetic alterations display most of the cardiac and pharyngeal arch defects observed in the 22q11.2 deletion syndromes. [11] TBX1 belongs to a family of transcription factors that contain a DNA binding domain called 'T-box'. Homozygous deletion of the Tbx1 gene in mice results in death in-utero. However, phenotypic features of 22q11.2 deletion were detectable in the embryos including abnormal facial features, thymic and parathyroid hypoplasia and cardiac abnormalities. Heterozygous mutants exhibited a less penetrant phenotype, with varying degrees of absence or reduction in fourth pharyngeal arches [61]. A lot of deletion experiments have been performed revealing that *Tbx1* has a specific time-dependent role in the formation of the pharyngeal pouches and their derivatives. In addition, these experiments revealed that *Tbx1* may very likely regulate the segmentation of the pharynx through the proliferative control of the endodermal epithelium thus influencing regular thymus organogenesis [11]. In fact individuals with chromosome 22q11.2 deletion syndrome frequently have a small hypoplastic thymus. Generally, individuals with an absent thymus and a profound T-cell lymphopoenia have been described as having complete DiGeorge syndrome, whereas most patients have a milder form of immunodeficiency and are described as having partial DiGeorge syndrome [67].

Infants with complete DiGeorge syndrome have absence of thymic function in addition to other defects of the third and fourth pharyngeal pouches [68, 69]. These profoundly immunodeficient infants represent less than 1% of patients with DiGeorge syndrome [53].

In the past, categorization of patients with DiGeorge syndrome as athymic required both profoundly low numbers of circulating T cells and very low T-cell proliferative responses to mitogens [64]. The size of the thymus correlates poorly with peripheral T-cell counts, suggesting that sources of extrathymic production of T cells might exist [70]. Evidence exists for the presence of microscopic remnants of thymic epithelial cells. One retrospective study showed the presence of retropharyngeal thymus tissue in children with features of DiGeorge syndrome [71]. Several centres have measured thymic function in patients diagnosed with DiGeorge syndrome on the basis of clinical features or of detection of the 22q11.2 deletion. Although a large proportion of patients has an absent or hypoplastic thymus at the time of cardiac surgery, most seem to have only a minor immune defect [53, 68, 72-74]. Most studies reported that patients show a reduction in the mean or median proportion and number of CD3+ T cells and CD4+ T helper cells compared with that of age-matched controls [72-77]. The function of T cells, as measured by incorporation of H3-thymidine to quantify lymphocyte proliferation after stimulation with mitogens, is generally normal [68, 72, 74, 75]. One additional feature is the expanded proportion of B cells (CD19+), natural killer T cells (CD16+CD56+) in patients compared with controls [72, 73, 76]. Although the rate of decline of T-cell numbers in patients with chromosome 22q11.2 deletion syndrome is slower than is that of controls, the T-cell population is smaller than is that of healthy controls throughout childhood [73, 75]. Notably, it was described that athymia can be present in patients with DiGeorge Syndrome who have significant numbers of T cells that can respond to mitogens [64]. For long time DiGeorge syndrome (DGS) was erroneously considered the human counterpart of the murine Nude phenotype. This association was based on the absence of the thymus leading to a similar immunological phenotype in both Nude mouse and DiGeorge patient. However, in-depth analysing these

two phenotypes, they appear more different. First of all, congenital alopecia and skin abnormalities, that represent the main features of the Nude/SCID phenotype, are absent in DGS. Moreover, even though they share the absence of the thymus, some immunologic aspects argue in favour of the Nude/SCID phenotype as the real model of human athymia. Thus, aim of this study was to identify differences in the T-cell ontogeny blockage in two different models of human athymic diseases. A particular attention will be paid to the comparison of T-cell pool maturation and functionality in Nude/SCID and DGS to define possible sites of extrathymic T-cell maturation.

Seven Nude/SCID patients and 14 DGS patients were included in this study as detailed in Table 1.

Table 1. Clinical and laboratory features of Nude/SCID and DGS patients

	Nude/SCID	DGS
Number of patients	7	14
Age (range)	16WG - 10 months	3 months – 17 years
Sex	5/7 F	8/14 F
Molecular alteration	6/7	14/14
Thymus alteration	7/7 Aplasia	11/14 Aplasia 3/14 Ipoplasia
Severe infections	5/7 *	4/14
Alopecia	5/7 *	Absent
Nail dystrophy	5/7 *	Absent
Cardiopathy	Absent	12/14
Ipoparatiroidism	Absent	7/14
Dismorfisms	Absent	9/14

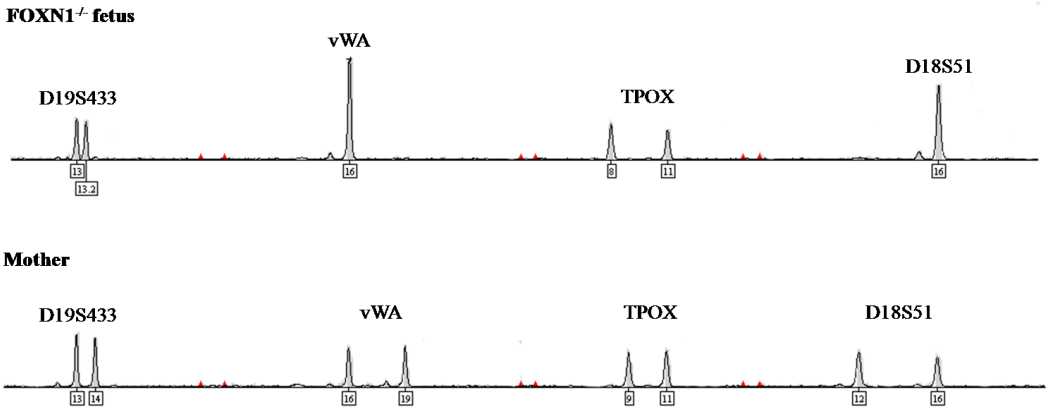
* Not determined in the fetuses

Results

Peripheral lymphocytes characterization of Nude/SCID and DGS patients

In order to analyze differences in peripheral cell pool between Nude/SCID and DGS patients, the presence of some cellular markers was evaluated through cytofluorimetry. Since one of the sample was obtained from cord blood of Nude/SCID fetus, first of all maternal contamination was excluded through STRs analysis on maternal and fetal DNA thanks to the collaboration of the Department of Medical Biochemistry and Biotechnology (DBBM)-CEINGE of "Federico II" University of Naples. As shown in Figure 1, the STR analysis of the cord blood revealed the presence of half of STR as maternal inherited thus excluding maternal contamination of the fetal sample.

Figure 1. STR analysis of cord blood



An extensive analysis of immunophenotype in these two groups of patients was performed. First of all the main lymphocytes subsets were

evaluated: stem cells (CD34+), T cells (CD3+), B cells (CD19+), NK cells (CD16+CD56+). The results are indicated in Table 2.

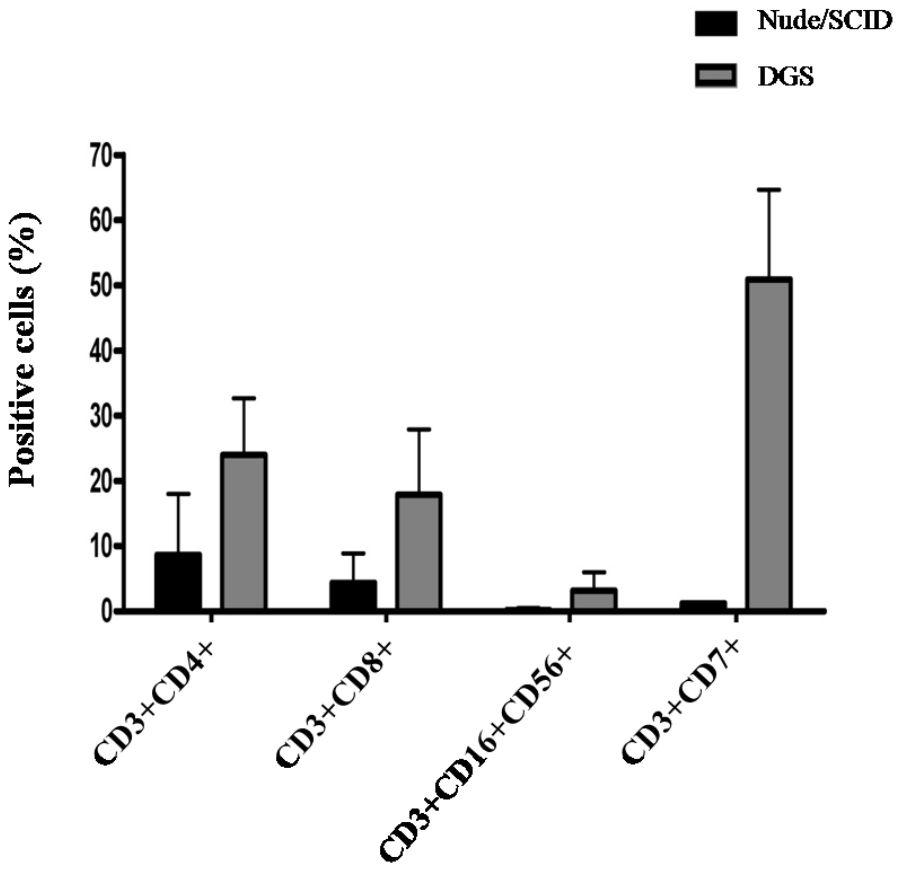
Table 2. Main lymphocytes subsets in Nude/SCID and DGS patients.

	Nude/SCID (%±SD)	DGS (%±SD)	p
Stem cells (CD34+)	1	0.039 ± 0.036	Not be evaluated
T cells (CD3+)	17.75 ± 12.97	46.85 ± 15.85	0.0041
B cells (CD19+)	60 ± 16.2	24.23 ± 9.85	0.0001
NK cells (CD16+CD56+)	24.33 ± 1.15	25.07 ± 12.87	0.93

Moreover, to further characterize the T lymphocytes detected in periphery, specific markers of T cells subsets were analysed: T Helper lymphocytes (CD3+CD4+), citotoxic T lymphocytes (CD3+CD8+), TNK lymphocytes (CD3+CD16+CD56+) and CD3+CD7+ lymphocytes. The values obtained are shown in Figure 2A.

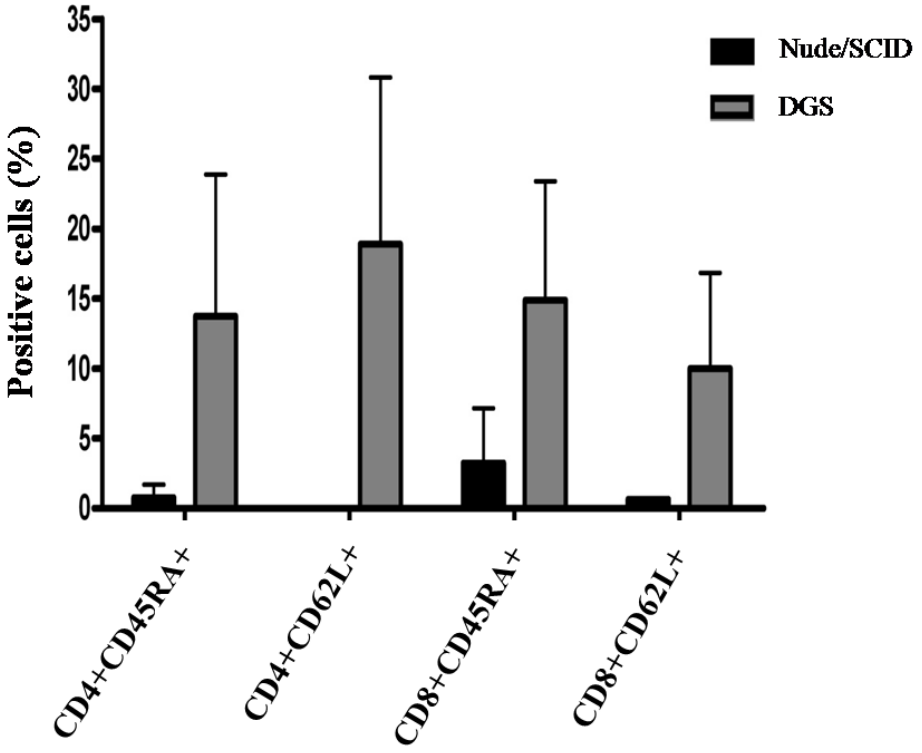
Figure 2. Nude/SCID and DGS patients immunephenotype

A) CD3+ cells characterization



Moreover, to evaluate, in the cord blood, the presence of naïve cells of both CD4+ and CD8+ cell compartment specific of thymic production, the following subsets were analyzed: CD4+CD45RA+, CD4+CD62L+, CD8+CD45RA+, CD8+CD62L+. The data obtained for these cell populations are showed in the Figure 2B.

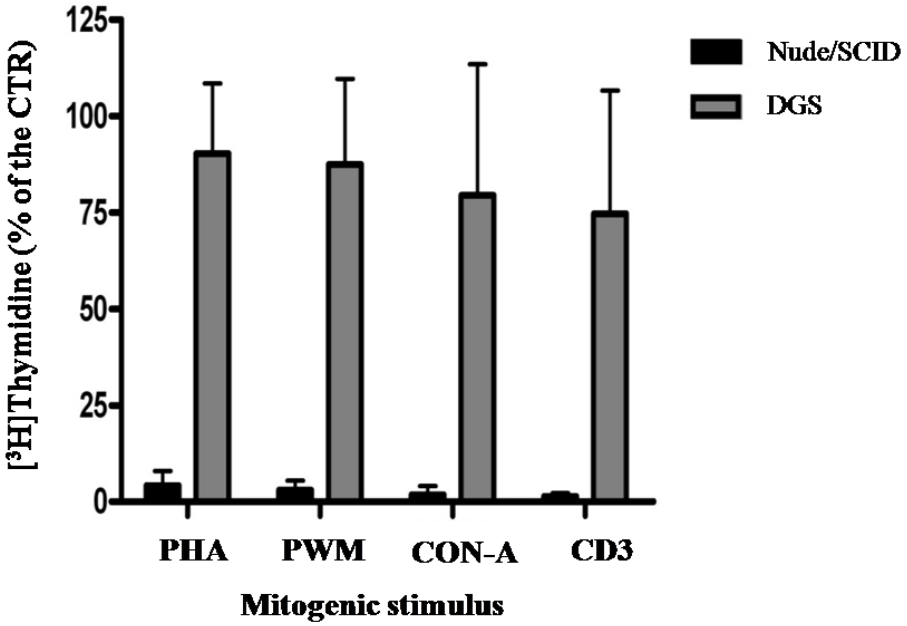
B) CD4+ and CD8+ naive lymphocytes



Evaluation of lymphocytes proliferation in Nude/SCID and DGS patients

In order to evaluate differences in the lymphocytes proliferation between Nude/SCID and DGS patients, the proliferation assay after mitogenic stimulation with PHA, PWM, CON-A and CD3 was performed. The results are indicated in the Figure 3.

Figure 3. Lymphocytes proliferation of Nude/SCID and DGS patients



§ 1.3 Conclusive remarks

Human Nude/SCID and DGS sindromes are characterized by severe anomalies of thymus development due to different genetic causes. Even thought these two syndromes share the absence of the thymus they appear quite different for both immunological and clinical phenotypes (see Table 3).

Table 3. Clinical and immunological features of Nude/SCID and DGS syndromes

Nude/SCID	DGS
Skin and skin annexa anomalies	**Cardiovascular defects**
Alopecia	Tetralogy of Fallot
Nail dystrophy	Type B interrupted aortic arch
	Truncus arteriosus
Athymia or absence of thymus function	Trasposition of the great arterias
	Aberrant right subclavian artery
Early onset of severe infections	Ventricular septal defects
Immunological abnormalities	**Glandular malformations**
T lymphocytes markedly reduced	Hipo/Aplasia of the thymus
(mainly T helper cells)	Hipo/Aplasia of the parathyroid glands
Increase of B lymphocytes	
Increase of Natural Killer cells	**Craniofacial anomalies**
Very low /absence of response to proliferation test	Cleft palate
	Micrognathia
	Ear anomalies
	Small mouth
	Behavioral disorders
	Learning difficulties
	Paranoid schizophrenia
	Major depressive illness
	Absence of opportunistic infections
	Immunological abnormalities
	Reduction of T lymphocytes
	Increase of B lymphocytes
	Increase of NK cells
	Normal response to proliferation test

Athymia in Nude/SCID syndrome is associated with a high susceptibility to infections leading to death in the first months of life. To date, the only therapeutical approach for these patients is represented by BMT, even though it is not able to recover the correct immune function. Contrary, in DGS patients the athymia is not associated to an increase susceptibility to infections and generally they died for the presence of cardiopathy. By an immunological point of view, the severe infections in Nude/SCID patients are due to a severe reduction of T lymphocytes, while in DGS patients they are mild and generally associated to a slow reduction of T lymphocytes. Most important, in Nude/SCID patients the lymphocytes are

not functional, while in DGS patients they normally respond to mitogenic stimulation in vitro thus justifying the lower infections in these patients than in Nude/SCID ones. Aiming to better characterize the differentiative blockage in these two different athymic diseases, the data obtained from this work revealed that in both Nude/SCID and DGS patients the CD34+ stem cells were normal, while CD3+ T lymphocytes were reduced in both, but with a more small aliquot in Nude/SCID patients than in DGS patients. The increase of B lymphocytes (CD19+) percentage in Nude/SCID patients was due to the reduction of T cells and it represented only a relative increase with no clinical implications. Contrary, in DGS patients the B lymphocytes were normal. The NK cells (CD16+CD56+) were normal in both syndromes. Thus, only T cell compartment was altered in both syndromes. Further analysis of T cells revealed that the CD3+CD4+ cells were reduced in both syndromes, but more in Nude/SCID patients. The number of CD3+CD8+ cells was normal in DGS patients, while it was reduced in Nude/SCID. However, in Nude/SCID syndrome, the reduction of CD3+CD8+ lymphocytes was lower than of CD3+CD4+ compartment thus implying that CD3+CD8+ cells can developed in a thymus independent manner. The evaluation of NKT cells (CD3+CD16+CD56+) revealed a reduction in Nude/SCID patients, while they were close to normal in DGS patients. Since CD7 molecule is expressed on early CD3+ cells and then it progressively disappeared on naïve and memory T cells [78], in both syndromes the CD3+CD7+ cells were analysed in order to evaluate the presence of immature cells developed in the absence of the thymus. The cytoflurimetric analysis revealed a markedly reduction of CD3+CD7+ cells in Nude/SCID patients, while in DGS they were close to normal. The presence of this cell population in DGS suggests the presence of an alternative pathway for the development of such cells.

By analysing the naïve compartment, no CD4+ naïve (CD4+CD45RA+, CD4+CD62L+) cells were detected in Nude/SCID patients, while they were present in DGS patients, even though in a small number. Also the analysis of the CD8+ naïve (CD8+CD45RA+, CD8+CD62L+) cells revealed the absence of these cell populations in Nude/SCID patients but the presence, even though under the normal values, of these cells in DGS patients.

Moreover, the evaluation of the proliferative capacity of the lymphocytes from both syndromes revealed a normal functionality in DGS patients opposite to what documented in Nude/SCID patients who didn't respond to mitogenic stimulation. This result is in keeping with the different clinical phenotype in the two syndromes, characterized by severe and untreatable infections in Nude/SCID patients.

Together these data indicate a severe reduction of both helper and citotoxic T cells in Nude/SCID patients, unlike DGS patients who only showed a slowly reduction of T cells which were also functional. Thus, even though these two syndromes share the athymia, their immunological phenotypes are completely different. Moreover, an in dept characterization of T cell compartment revealed, in DGS, the presence of different cell subsets which were poorly represented in Nude/SCID. All together, these observations support the hypothesis that an alternative site could ensure T cell production in absence of the thymus. As it is clear from our data, this site of extrathymic T cell development is probably altered in Nude/SCID syndrome since no mature T cells were found. This hypothesis is strongly supported by other data on NKT (CD3+CD16+CD56+) cells and on CD3+CD7+ cells. In particular, NKT cells are considered of thymic origin, but since they were also found in liver, spleen and bone marrow and in athymic mice, they are supposed to develop outside the thymus, probably in the liver [79]. Thus, the presence of these cells in the DGS patients is

probably due to an extrathymic site of lymphocytes development which was absent in the Nude/SCID syndrome. Moreover, also the presence of the CD3+CD7+ cells, probably representing precursor cells, argues in favour of a population developing outside the thymus in that, in the mouse, a cell population CD2+CD3+CD7+ probably of intestinal origin, was documented [80].

The main difference between Nude/SCID and DGS is represented by the presence of alopecia and skin keratynocytes alterations in Nude/SCID, absent in DGS. These abnormalities are due to the absence of the transcription factor FOXN1 responsible for Nude/SCID syndrome, which is expressed both in thymic and epithelial cells. So, since the main difference between these two syndromes is represented by skin abnormalities, it was supposed that the skin, which is normal in DGS, can act as a surrogate organ capable to support T cell differentiation in the absence of the thymus. This hypothesis is supported by the observation that cellular colture containing skin cells expressing FOXN1 and hematopoietic precursor cells are able to support lymphocyte differentiation in vitro [81].

In conclusion, the data emerging from this study lead to consider the Nude/SCID syndrome as real model of human athymia in that in DGS it was not documented a real differentiative blockage of T cell development.

CHAPTER II

Immunodeficiencies due to alteration of the cerebellar function: the Ataxia Telangiectasia

Among those PIDs whose causing genes are not selectively expressed in the hematopoietic compartment there is Ataxia-telangiectasia (A-T), a rare autosomal recessive disorder, characterized by progressive neurological dysfunction, especially affecting the cerebellum, oculo cutaneous telangiectasia, high incidence of neoplasms and hypersensitivity to ionizing radiations [82, 83].

Immunodeficiency is present in 60-80% of individuals with A-T, it is variable and do not correlate well with the frequency, severity or spectrum of infections [84]. The immunodeficiency is progressive and the most consistent immunodeficiency reported is poor antibody response to pneumococcal polysaccharide vaccines [84]. Moreover, serum concentration of the immunoglobulins IgA, IgE and IgG2 may be reduced. Approximately 30% of individuals with A-T who have immunodeficiency have T-cell deficiencies. At autopsy, virtually all individuals have a small embryonic-like thymus. Unlike most immunodeficiency disorders, the spectrum of infection in individuals with A-T does not comprise opportunistic infections. Some individuals develop chronic bronchiectasis and the frequency and severity of infections correlates more with general nutritional status than with the immune status. Individuals with frequent and severe infections appear to benefit from intravenous immunoglobulin (IVIG) replacement therapy [84]; however, longevity has increased substantially even in individuals not receiving IVIG. Immunological abnormalities mainly

include impaired antibody responses, T-cell lymphopenia, primarily affecting CD4+ lymphocytes, and impaired proliferative response to mitogens and antigens. However, severe infections are uncommon in A-T [85]. On the contrary, clinical signs related to the progressive neurodegeneration are overwhelming and dramatically affect the quality of life. Purkinje cells are thought be selectively depleted, thus resulting in the progressive cerebellar atrophy of the cortex associated with significant thinning of the molecular layer, as revealed by autopsy and biopsy studies [86, 87].

A-T is caused by mutation of A-T mutated gene product (ATM) [83]. ATM is a protein kinase, that regulates those responses required for cell survival in response to DNA double-strand breaks (DSBs) caused by ionizing radiation, DNA damage agents and DNA recombination [88].

Unfortunately, currently there is no effective treatment for A-T, but supportive care of neurological symptoms, as physical, occupational and speech/swallowing neurorehabilitation. Eventually, the progressive neurodegeneration and pneumonia are a frequent cause of death in patients with A-T.

§ 2.1 In Ataxia Telangiectasia patients betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity

The central nervous system is particularly vulnerable to oxidative stress due to its high rate of metabolism and to the disproportionately low levels of its oxidative defence mechanisms [86]. The increased and unopposed ROS production can lead to neurotoxicity those results in neural damage and eventually in cell death.

Even though A-T is a non-curable disease, recently, Broccoletti et al. demonstrated a dramatic improvement of cerebellar functions in a few A-T patients after a short-term betamethasone therapy [87]. The highest clinical

improvement was on speech disturbance, stance, and finger chase items [87]. As the effect of betamethasone was transient in nature, it should be argued that the intimate mechanism of this pharmacological action regards some still unidentified steroid-dependent biochemical event. This is not surprising, because a permanent reversion of the molecular alteration is not expected, and the drug presumably acts on the damage derived from ATM dysfunction. Of note, ATM protein, after its autophosphorylation, activates multiple substrates involved in cell cycle regulation, such as p53 and, eventually, leads, cooperating with other DNA repairing factors, to cell cycle arrest. ATM is also involved in sensing and modulating intracellular redox status, even though it is not clear whether ATM itself is directly involved in ROS production [89, 90]. However, ATM deficiency causes oxidative damage to proteins and lipids in brain, testes and thymus. Cells have developed a wide array of protective mechanisms against ROS, including small reducing molecules, antioxidative enzymes and damage/repair systems [85]. One of the small reducing molecules is glutathione (GS), which appears in the cells in both its oxidized and reduced (GSH) forms. It works in detoxifying specific ROS by itself or in combination with other enzymes, such as SOD, which converts superoxide radicals into H_2O_2 and O_2, catalase, glutathione peroxidase (PHGPx) and glutathione-S-transferases. Oxidants overproduction and/or dysfunction of endogenous antioxidant defenses result in oxidative stress induced injury with damage to all the major classes of biological macromolecules, such as nucleic acids, proteins, lipids and carbohydrates. The CNS is particularly vulnerable to oxidative stress due to its high rate of metabolism and to the disproportionately low levels of its oxidative defense mechanisms [86]. The increased and unopposed ROS production can lead to neurotoxicity that results in neural damage and eventually in cell death.

Due to the importance of the topic and since the intimate molecular mechanism by which betamethasone led to this effect remains unclear, aim of this study was to evaluate whether the beneficial effect of betamethasone therapy could be mediated by interference in ROS generation/neutralization process. Thus, in this part of my study, we measured in a small cohort of A-T patients direct markers of ROS production, during a short-term steroid treatment.

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In ataxia-teleangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity

I. Russo[a], C. Cosentino[b], E. Del Giudice[a], T. Broccoletti[a], S. Amorosi[a], E. Cirillo[a], G. Aloj[a], A. Fusco[a], V. Costanzo[b] and C. Pignata[a]

[a]*Department of Pediatrics, "Federico II" University, Naples, Italy; and* [b]*Genome Stability Unit, London Research Institute, Clare Hall Laboratories, South Mimms, London, UK*

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Background and purpose: Ataxia-telangiectasia (A-T) is a rare autosomal recessive disorder caused by alterations of the A-T mutated (ATM) gene. Although A-T is a noncurable disease, we, previously, documented a clear improvement of cerebellar functions during a short-term betamethasone trial. The aim of this study was to define the underlying biochemical mechanism.

Methods: In six A-T patients receiving a short-term steroid therapy, intracellular glutathione (GSH) levels were evaluated with a colorimetric assay. The lipid peroxidation level and reactive oxygen species (ROS) production were evaluated using commercial assays. All the parameters were compared with the improvement of cerebellar functions expressed as delta (Δ) of the Scale for the Assessment and Rating of Ataxia (SARA).

Results: We observed an inverse correlation between Δ SARA and the severity of cerebellar atrophy and between the latter and basal GSH values. Four of the five patients with the highest Δ SARA also had the highest GSH values. Moreover, even though basal ROS values were comparable in patients and controls, in the only patient studied at different time-points of therapy, a remarkable reduction in ROS levels was documented.

Conclusion: We suggest that antioxidative mechanisms play a role in favouring the improvement of cerebellar functions observed in A-T patients receiving a short-term betamethasone trial.

Introduction

Ataxia-telangiectasia (A-T) is a human genetic disease whose hallmarks are neurodegeneration, immunodeficiency, genomic instability and cancer predisposition [1]. A-T is caused by mutation of A-T mutated gene product (ATM) [2]. ATM is a protein kinase, that regulates those responses required for cell survival in response to DNA double-strand breaks (DSBs) caused by ionizing radiation, DNA damage agents and DNA recombination [3]. ATM protein, after its autophosphorylation, activates multiple substrates involved in cell cycle regulation, such as p53 and, eventually, leads, cooperating with other DNA repairing factors, to cell cycle arrest. ATM is also involved in sensing and modulating intracellular redox status, even though it is not clear whether ATM itself is directly involved in reactive oxygen species (ROS) production [4,5]. However, ATM deficiency causes oxidative damage to proteins and lipids in brain, testes and thymus. Furthermore, ATM-deficient mice exhibit genomic instability and hypersensitivity to ionizing radiation and other treatments that generate ROS [6].

In keeping with this, in ATM-deficient mice the overexpression of superoxide dismutase 1 (SOD), an enzyme involved in hydrogen peroxide production, exacerbated certain features of the A-T phenotype [7]. These observations point to additional roles for ATM in cellular metabolism other than its direct role in the response to DSBs induction, such as its involvement in ROS balance.

Cells have developed a wide array of protective mechanisms against ROS, including small reducing

Correspondence: Claudio Pignata, MD, PhD, Department of Pediatrics, Unit of Immunology,"Federico II" University, via S. Pansini 5-80131, Naples, Italy (tel.: +39 081 7464340; fax: +39 081 5451278; e-mail: pignata@unina.it).

molecules, antioxidative enzymes and damage/repair systems [8]. One of the small reducing molecules is glutathione (GS), which appears in the cells in both its oxidized and reduced (GSH) forms. It works in detoxifying specific ROS by itself or in combination with other enzymes, such as SOD, which converts superoxide radicals into H_2O_2 and O_2, catalase, glutathione peroxidase (PHGPx) and glutathione-S-transferases. Oxidants overproduction and/or dysfunction of endogenous antioxidant defenses result in oxidative stress induced injury with damage to all the major classes of biological macromolecules, such as nucleic acids, proteins, lipids and carbohydrates. The central nervous system is particularly vulnerable to oxidative stress because of its high rate of metabolism and to the disproportionately low levels of its oxidative defense mechanisms [9]. The increased and unopposed ROS production can lead to neurotoxicity that results in neural damage and eventually in cell death.

Even though A-T is a noncurable disease, recently, we demonstrated an improvement of cerebellar functions in a few A-T patients after a short-term betamethasone therapy [10]. Aim of this study is to evaluate whether the beneficial effect of betamethasone therapy could be mediated by interference in ROS generation/neutralization process. Thus, we measured in this small cohort of A-T patients direct markers of ROS production, during a short-term steroid treatment.

Subjects and methods

Patients

Blood samples were obtained from six consecutive patients (three males), of 5, 6, 14, 15, 27 and 29 years of age, respectively (mean age 16.3 years; range 5–29 years), affected with A-T. The patients, upon written consent, received a cycle of oral betamethasone at the dosage of 0.1 mg/kg/day for 10 days divided every 12 h. No antioxidants, as *N*-acetyl cysteine, vitamin E or alphalipoic acid, were given to the patients during the 2 years preceding the trial. The clinical evaluations were carried out before therapy (T0), 48 h from the beginning (T48), at the end of the therapy (T10d) and, eventually, 7 days from the withdrawal (T7 off therapy). The neurological evaluation was performed through the Scale for the Assessment and Rating of Ataxia (SARA) (see appendix E1 on the neurology Web site at http://www.neurology.org/cgi/cintent/full/66/11/1717/DC1) [10]. In particular, in this study we utilized the variation in SARA Score (Δ), considering that the higher Δ is the higher amelioration. Cerebellar atrophy score was calculated as follows: 0 score, absence of cerebellar atrophy; 1 score, absence of cerebellar atrophy with moderate pontocerebellar angle cisterns enlargement; 2 score, moderate atrophy involving mostly both superior and inferior portion of the vermis and, at a lesser extent, the cerebellar hemispheres with moderate enlargement of periquoral spaces; 3 score, severe atrophy of superior portion of vermis and moderate atrophy of inferior part of vermis; severe atrophy of superior and lateral portion of cerebellar hemispheres and moderate atrophy of inferior hemispheres; 4 score, global and severe atrophy of the superior and inferior part of vermis and the whole cerebellar hemispheres with marked fourth ventricle enlargement.

Cell isolation and treatment

Peripheral blood mononuclear cells (PBMC) were obtained from A-T patients and normal donors heparinized peripheral blood by Ficoll-Hypaque (Cambrex, Milan, Italy) density gradient centrifugation. Cells were maintained in RPMI 1640 (Cambrex) supplemented with 10% FBS (Cambrex), 2 mM/l L-glutamine (Invitrogen Life Technologies, Milan, Italy), and 50 μg/ml gentamicin (Invitrogen Life Technologies), and cultured at 37°C, 5% CO_2 without stimuli or stimulated for 48 h with phytohemagglutinin (PHA) (8 μg/ml) and then collected.

Determination of lipid peroxidation

Cells (0.8×10^5) were lysed by freeze and thaw in 10 mM TRIS pH 7.5 and the lipid peroxides were assessed with Cayman Chemical Company assay kit (Ann Arbor, MI, USA), according to the manufacturer's instruction. This kit measures malonaldehyde and 4-hydroxyalkenals that result from peroxidation of polyunsaturated fatty acids. The samples were normalized for cell number.

Evaluation of intracellular glutathione

The cells were lysed by freeze and thaw. Cellular levels of GSH were determined using DIGT-250 GSH colorimetric assay kit (BioAssay Systems, CA, USA). Cells (0.8×10^5) were treated for 48 h with various agents and collected by centrifugation at 1300 *g* for 10 min at 4°C. All the samples were analysed following manufacturer's instructions. Optical density (OD)-values were read at 412 nm. The samples were normalized for cell number.

Measurement of intracellular ROS

The cellular ROS accumulation was measured using the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA)

method. DCFH-DA is a nonfluorescent compound, and it can be enzymatically converted to highly fluorescent compound, DCF, in the presence of ROS [11]. After and before exposure to betamethasone, 1×10^6 cells were treated with 5 μM DCFH-DA at 37°C for 20 min, at 5% CO_2, washed with PBS, collected and analysed immediately on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an argon laser emitting at 488 nm. CELL QUEST software (Becton Dickinson) was used to acquire and evaluate all the events.

Results

Evaluation of the relationship between GSH levels and neurological amelioration during steroid therapy

Glutathione (GSH) is a prominent reducing molecule that is implicated in the first line of defense from oxidative stress with other antioxidants or scavenger proteins as vitamin E (α-tocopherol), vitamin C (ascorbic acid), uric acid, carotene [5]. In our previous work, we reported an indirect correlation between age and improvement of cerebellar functions. As shown in Fig. 1a, an indirect correlation between the extent of improvement of cerebellar functions during betamethasone treatment, evaluated through Δ SARA Score and the severity of cerebellar atrophy was observed ($R = -0.873$; $P < 0.05$). We next evaluated the correlation between cerebellar atrophy and basal GSH levels in unstimulated cells. An indirect correlation between these two parameters was noted ($R = -0.07344$; $P < 0.05$) (Fig. 1b).

The existence of a link between basal GSH levels and Δ SARA Score was also evaluated. Of note, a trend to a correlation between improvement of cerebellar functions during the therapy and GSH levels at T0 was noted. In fact, four of five patients, who had a significant clinical amelioration (Δ SARA Score ≥4), had the highest GSH values (0.084, 0.102, 0.086 and 0.079 OD-values respectively). The only patient (P6) who did not have any improvement of cerebellar functions had negligible GSH value (0.011 OD-value).

As in the physiology of immune response, lymphocytes are committed to proliferate in response to antigenic stimulation. We previously documented that the proliferative response to PHA decreased during steroid treatment. Thus, in this study we evaluated the effect of PHA stimulation on the GSH levels and observed that in five of six patients PHA stimulation induced a significant decrease of GSH levels, suggesting that the lower proliferative capability parallels a reduction of the protective GSH system (Fig. 2).

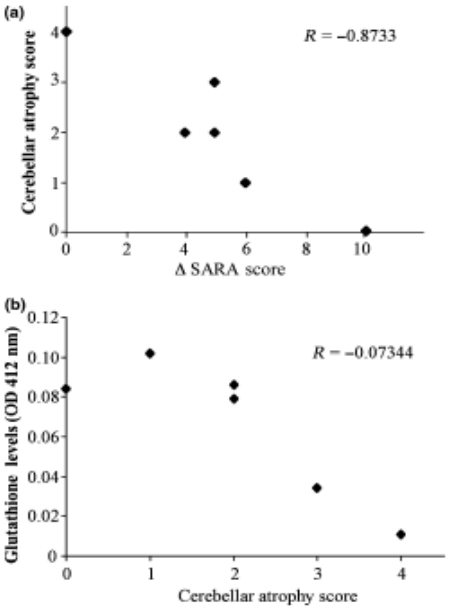

Figure 1 Correlation between neurological amelioration during steroid therapy, cerebellar atrophy and glutathione levels. (a) Indirect correlation between cerebellar atrophy score and the improvement of cerebellar functions, expressed as Δ SARA score, in ataxia-telangiectasia patients. (b) Indirect link between basal glutathione levels, evaluated by DIGT-250 colorimetric assay and cerebellar atrophy score.

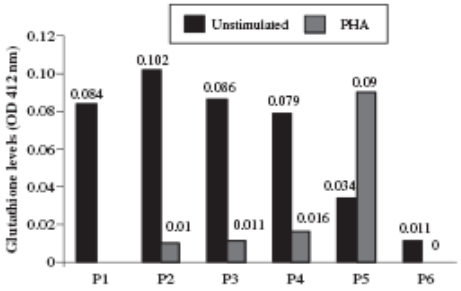

Figure 2 Cellular levels of glutathione in peripheral blood mononuclear cells, unstimulated or stimulated with PHA, obtained from ataxia-telangiectasia patients. In five of six patients PHA stimulation induces a significant decrease of glutathione levels.

The effect of ROS hyper-production on lipid peroxidation

To further evaluate the role of oxidative injury in A-T, we also have quantified the lipid peroxidation as a direct effect of a potential abnormal ROS production. Lipid peroxidation results in the formation of highly reactive and unstable hydro-peroxides of both saturated and unsaturated lipids. In our study, lipid peroxidation levels were not detectable in five of six patients either before or during the therapy (data not shown). Only the patient (P6), who had lipid peroxidation levels above the threshold of detection (detection limit 0.14 nm), did not show any clinical response to steroid therapy.

Evaluation of intracellular ROS levels in lymphocytes

Before entering into the study, we evaluated in all patients the DCF-DA, a direct marker of oxidative status, to define whether the clinical response to therapy was correlated with the pretreatment ROS levels. DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolysed to the nonfluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein DCF. No significant difference between patients and controls in ROS levels before therapy was observed and no correlation between ROS levels and the clinical response to treatment was noted. In one patient (P1) the determination of ROS was carried out at all time-points of the clinical trial. As depicted in Fig. 3, although ROS values at T0 were comparable to the controls, a substantial decrease in ROS during the steroid therapy was observed, reaching half of the starting value. Of note, this patient was the patient who exhibited the highest improvement of cerebellar functions during therapy.

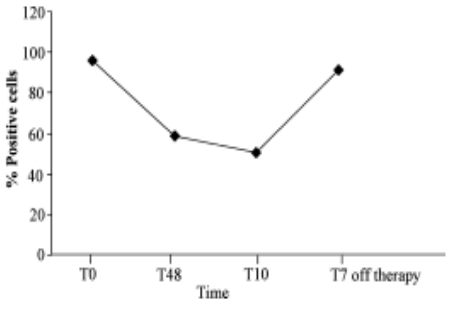

Figure 3 Intracellular reactive oxygen species (ROS) levels in peripheral blood mononuclear cells derived from the better clinical responder (P1) to betamethasone treatment. A substantial decrease in ROS levels is observed during steroid therapy.

Discussion

Previously, we observed in a small cohort of A-T patients an improvement of cerebellar functions evaluated through the SARA scale during a short-term betamethasone treatment [10]. This improvement was inversely correlated with the age and severity of cerebellar atrophy. Here, we report that patients with the more severe cerebellar atrophy had the lowest GSH levels. GSH is a molecule implicated in the first line of defense from ROS production along with other protein scavengers or low molecular weight antioxidants. Neuronal antioxidant defenses mainly rely on the cellular levels of GSH [12–14]. In particular, as matter of fact, the main aim of this study is to compare the clinical amelioration, defined as Δ Sara Score, with cerebellar atrophy and anti-oxidative capacity of the cells, measured through the GSH evaluation.

There is evidence indicating a direct relationship between excessive ROS production and the pathogenesis of A-T. It has been documented that in ATM knockout mice, GSH levels were significantly higher in the cerebellum, as a compensatory mechanism induced by over-production of ROS [6]. Of note, in this study we observed that the highest basal GSH values were present in PBMC from those patients who better responded to the betamethasone therapy. Several lines of evidence indicate that neurons degeneration parallels T-cell loss, thus implying that both systems share common transcription factors, receptors and cytokine signalling molecules. Thus information obtained on PBMC may also be relevant to understand cerebellar pathology [15]. However, we couldn't demonstrate that ROS levels were higher in patients than in controls. This is not surprising in that in a previous study no significant increase in oxidative stress biomarkers was found in A-T patients [16]. The comparable ROS levels between patients and controls do not necessarily rule out that the effect of betamethasone is because of interference on ROS homeostasis. Even though no conclusive data are available on a direct effect of steroids on ROS levels, there is evidence suggesting opposite effects in different systems. In platelets, an inhibitory effect of glucocorticoids in the generation of ROS has been documented [17]. Similarly, in mononuclear cells steroids have been shown to exert their anti-inflammatory role also by down-modulating ROS levels [18]. On the contrary, an increase in ROS production has been observed in endothelial cells and considered involved in the pathogenesis of steroid-induced hypertension [19].

It should be noted that in the only patient studied at different time-points for intracellular ROS levels, betamethasone therapy induced a significant reduction in ROS levels. It is interesting to note that the behaviour

of ROS levels in this patient, who better responded to the treatment, correlated with the clinical improvement. In particular, it is remarkable that the ROS levels decreased during the phase of the improvement and returned to normal 7 days off the therapy, paralleling the clinical worsening. This finding confirms that steroid induced improvement of cerebellar function is drug dependent and not long-lasting, even though this issue requires a further *ad hoc* study due to the importance of potential clinical implications. Furthermore, we found that lipid peroxidation occurred only in the patient who did not exhibit any clinical improvement during the therapy and, importantly, that it was associated with the most severe cerebellar atrophy.

As for the molecular mechanisms of action of betamethasone, several lines of evidence indicate that steroids have remarkable effects through both genomic and nongenomic mechanisms, the latter well documented also in neural system [20]. In our study, we also examined the modification of GSH levels after the induction of massive oxidative stress through PHA stimulation. The reduction of GSH level observed in five of six patients supports the hypothesis that the GSH protective antioxidant apparatus represents the major limiting factor in the maintenance of redox homeostasis in A-T PBMC.

Another possible explanation, however, is that the lowest GSH levels found in patients with most severe cerebellar atrophy are only related to the extent of cerebellar damage rather than to the response to therapy. With this regard, we do not have a clear demonstration that betamethasone really exerts a disease modifying role rather than only a symptomatic effect.

These findings suggest that the antioxidative protective glutathione system plays a role in the protection from the cerebellar atrophy and may be implicated in the improvement of cerebellar functions during short-term betamethasone therapy. The importance of our data mostly relies on the absence of a curative therapy for A-T at present.

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§ 2.2 Conclusive remarks

Even though A-T is non-curable Broccoletti et al. demonstrated an improvement of the cerebellar functions in 6 A-T patients after a short-term betamethasone therapy (0.1 mg/kg/day for 10 days) [87]. Although the clinical response to betamethasone was evident, there was no evidence indicating the intimate mechanism of action of this drug in A-T.

In this study, on the basis of our finding, we concluded that betamethasone response in A-T patients is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. The importance of this study relies principally on the severity of the neurological signs. In fact, this childhood disease is characterized by progressive impairment of gait and speech, oculomotor apraxia (inability to move the eyes from one object to another), oculocutaneous telangiectasia (dilated blood vessels), cerebellar atrophy, sterility and radiosensitivity [91]. A-T is primarily a syndrome of progressive cerebellar ataxia but more diffuse changes to the CNS are also evident. Cerebellar degeneration in A-T manifests as dystrophic changes involving the dendrites and axons of Purkinje cells and ectopic Purkinje cells are evident. Of all the features of A-T the progressive cerebellar neurodegeneration is the most debilitating leading to peripheral neuropathy and eventually to spinal muscular atrophy.

Thus, any effective treatment for A-T would ideally involve prevention or at least slowing of the progressive neurodegeneration. As mentioned above, at present there is no therapy available to cure or prevent the progress of A-T, but it is possible to alleviate some of the symptoms associated with immunodeficiency and deficient lung function but neither the cancer predisposition nor the progressive neurodegeneration can be prevented.

There is no cure for the progressive neurodegeneration with conventional therapies but some promise exists. Most importantly, the

rescue of the neurological function, at some extent, suggests that cell loss is an ultimate feature in A-T and those biochemical alterations, presumably reversible, precede for a long time cerebellar atrophy.

This study on the implicated mechanisms may open an important window on novel therapeutic agents capable of controlling neurological symptoms in A-T.

CHAPTER III

Immunodeficiencies due to alteration of genes expressed in hematopoietic cells

Several molecules, involved in the intracellular communication network, have been identified as the cause of many forms of SCID. In most cases, unlikely from the above described SCIDs, these molecules are exclusively expressed in hematopoietic cells, being involved in cell development and/or functionality of terminal differentiated cells of immune system.

Defects of cytokine-mediated signaling are responsible for the majority of SCID in humans affecting both cell development and functionality of differentiated cells. SCIDX1 accounts for 40% of all cases of SCID and is caused by mutations of the IL-2 receptor g gene (IL2RG), which encodes for the common γ chain (γc) shared by cytokine receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In particular, IL-7 mediates expansion of early thymocyte progenitors, whereas IL-15 plays a role in NK cell development [92]. Accordingly, patients with SCIDX1 lack both T and NK cells, whereas they have a normal number of circulating B lymphocytes [93]. The γc is physically and functionally coupled to the intracellular tyrosine kinase Janus kinase (JAK) 3, which delivers γc-mediated intracellular signaling. Hence defects of JAK3 result in an autosomal recessive form of SCID with an immunologic phenotype undistinguishable from that of SCIDX1 [94]. Mutations of the IL7R gene (encoding for the α chain of the IL-7 receptor) abrogate T-lymphocyte development but leave B-cell and NK-cell development intact [95].

Among the immunodeficiencies due to alterations of functionality of lymphocytes, there is the Hyper IgE syndrome (HIES). It is a rare primary immunodeficiency characterized by the triad of elevated IgE and eosinophilia, eczema, and recurrent skin and pulmonary infections [96]. Dominant negative mutations in the signal transduction and activation of transcription (STAT) 3 gene account for the vast majority of autosomal dominant (AD) and sporadic cases, but the pathogenesis of some of the varied clinical features of HIES remains poorly understood [97, 98]. Autosomal recessive diseases with elevated IgE that have been grouped with HIES are considerably more rare than the autosomal dominant STAT3-deficient form of HIES. They are clinically distinct, more severe, and may be associated with autoimmunity and vasculitis [99]. Specific mutations have not been identified in these patients. However, a single case of homozygous mutation leading to a premature stop in Tyk2 was found [100]. Tyk2 is a key signal transduction molecule upstream of STAT3 and therefore critical for the production of IL-6, IL-10, IL-12, IL-23, and IFN-α, many of the same cytokines involved with STAT3.

§ 3.1 Alteration of IL-12R signalling in children with high serum level of IgE

The immunophenotype of HIES has long implicated defects in both innate and adaptive immune responses, leading to the suspicion that cytokines and chemokines, which are involved in all aspects of the immune response, were likely to be involved in the pathogenesis of HIES [96]. Cytokines are produced in response to diverse cellular stimuli and serve as growth factors and regulators of the immune response [96]. An appropriate immune response mostly relies on a well orchestrated Th1/Th2 dichotomy, whose hallmark is based on the capability of the individual subset to work in an autocrine fashion leading to amplify its own cell development and to

cross-regulate the other subset development and activity [101, 102]. It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance [103]. In particular, Th1 response is induced by IL-12 produced by B cells and macrophages [104]. The receptor is up-regulated during T-cell activation and IL-12Rβ2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rβ1 is constitutively expressed in resting cells [105]. The transducing element of the receptor is the IL-12Rβ2 chain that functionally interacts with members of the family of Signal Transducers and Activators of Transcription (STAT), and in particular STAT4 [106]. This transcription factor is promptly phosphorylated on tyrosine residues upon receptor triggering [106]. Th1 cells develop in the presence of IL-12 and STAT4 signaling and secrete mainly IFN-γ [107]. Moreover, experimental evidence using the knock-out technology supports the concept that IL-12R/STAT signaling pathway plays a role for the induction of a Th1 response [108, 109]. Thus, by studying children with elevated serum IgE levels divided on the basis of the IgE values (> 2000 kU/l or < 2000 kU/l) the aims of this part of the study were to investigate at a functional level whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels, and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus.

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Altered signaling through IL-12 receptor in children with very high serum IgE levels

Anna Fusco, Ilaria Vigliano, Loredana Palamaro, Emilia Cirillo, Giuseppina Aloj, Giovanna Piscopo, Giuliana Giardino, Claudio Pignata *

Department of Pediatrics, "Federico II" University, Naples, Italy

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ABSTRACT

An alteration of Th1/Th2 homeostasis may lead to diseases in humans. In this study, we investigated whether an impaired IL-12R signaling occurred in children with elevated serum IgE levels divided on the basis of the IgE levels (group A: >2000 kU/l; group B: <2000 kU/l). We evaluated the integrity of the IL-12R signaling through the analysis of phosphorylation/activation of STAT4, and mRNA expression and membrane assembly of the receptor chains. At a functional level, a proliferative defect of lymphocytes from group A patients was observed. In these patients, an abnormal IL-12R signaling was documented, and this finding was associated with abnormal expression of the IL-12Rβ2 chain. Our data indicate that in patients with very high IgE levels the generation of Th1 response is impaired, and that this abnormality associates with abnormal IL-12R signaling.

1. Introduction

The identification of distinct CD4+ T helper cells (Th1 and Th2) exerting peculiar functions and differing on the basis of the production of a unique cytokine profile greatly contributed to our understanding of the intimate mechanism implicated in the different type of host immunity. Th1 cells produce interferon (IFN)-γ and interleukin (IL)-2 and, predominantly, promote cell-mediate immune responses, whereas Th2 cells that produce IL-4, IL-5 and IL-13 provide help for some B cell responses as IgG1 and IgE production [1,2]. Overall, an appropriate immune response mostly relies on a well orchestrated Th1/Th2 dichotomy, whose hallmark is based on the capability of the individual subset to work in an autocrine fashion leading to amplify its own cell development and to cross-regulate the other subset development and activity [3,4]. It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance. Moreover, the susceptibility to infections by certain pathogens is associated with low levels of IFN-γ [5]. Thus, alteration of Th1/Th2 homeostasis, also involving further regulatory T cells as Th17, may lead to diseases in humans [3,6]. A Th1 response is implicated under ordinary circumstances in resistance to several intracellular pathogens, but an excessive Th1 response is associated with different autoimmune diseases, as rheumatoid arthritis [7,8], type I diabetes [9] or multiple sclerosis [10]. On the contrary, a Th2 dominated response, usually involved in the response to extracellular pathogens as parasitic or helminths, is associated with allergic disorders and the progression of chronic infections as AIDS [11].

The dimeric cytokine IL-12, produced by B cells and macrophages, plays a pivotal role for the induction of a Th1 response [12]. Its cloned receptor consists of two subunits, IL-12Rβ1 and β2, both required for high affinity binding to IL-12 and full cytokine responsiveness [13]. The receptor is up-regulated during T-cell activation and IL-12Rβ2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rβ1 is constitutively expressed in resting cells [14]. The transducing element of the receptor is the IL-12Rβ2 chain that functionally interacts with members of the family of Signal Transducers and Activators of Transcription (STAT), and in particular STAT4 [15]. This transcription factor is promptly phosphorylated on tyrosine residues upon receptor triggering [15]. Th1 cells develop in the presence of IL-12 and STAT4 signaling and secrete mainly IFN-γ [16]. Moreover, experimental evidence using the knock-out technology supports the concept that IL-12R/STAT signaling pathway plays a role for the induction of a Th1 response [17,18].

The aims of our study were to investigate at a functional level whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels, and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus. In particular, the activation of STAT4 molecule that follows IL-12R triggering, and the analysis of gene transcription and membrane assembly of the receptor itself were investigated in allergic children divided on the basis of the amount of serum IgE.

* Corresponding author. Address: Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini, 5-80131 Naples, Italy. Fax: +39 081 545 1278.
E-mail address: pignata@unina.it (C. Pignata).

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2. Materials and methods

2.1. Subjects

Twenty patients with elevated IgE levels and history of allergy were enrolled into the study. Sixteen patients were affected by asthma, 3 of them also by rhinitis, and 4 had a history of atopic dermatitis. The patients divided in two subgroups on the basis of IgE levels: group A consisted of 10 patients, 10 males, range of age 5–15 years, with very high serum IgE levels (>2000 kU/l, range 2152–5000 kU/l); group B consisted of 10 patients, 9 males, range of age 6–15 years, with high serum IgE levels (IgE value between the age specific mean ± 2 SD and 2000 kU/l, range 93–1152 kU/l) (Table 1). Twenty healthy controls, 16 males range of age 6–15 years (IgE range 85–100 kU/l), were also studied. Informed consent was obtained when required. All patients enrolled into the study did not receive any treatment, including steroid or non-steroid drugs, in the month before entering into the study. No difference was found between group A and B in either the number per year or the severity of allergic manifestations. In all patients, the clinical features persisted for more than 2 years.

The Hyper-IgE Syndrome (HIES) was excluded by the absence of typical clinical and immunological features according to the clinical score for HIES (Table 1) [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed. Other conditions accompanied by elevated serum IgE concentration, including AIDS, helminths and parasitic infections were also excluded by clinical and laboratoristic features.

The study has been approved by the Institutional Review Board.

2.2. Cell culture and proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation by standard procedure and cultured in triplicates (2×10^5/well). Cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories, Detroit, MI), phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co., St. Louis, MO). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody (Ortho Diagnostic, Raritan, NJ). To evaluate allogeneic response, patients responder cells (1×10^5) were stimulated with an equal amount of irradiated stimulator cells from controls in a standard one-way mixed lymphocyte reaction assay. Cell mixtures were cultured in 96-well round-bottom microtiter plates (Becton Dickinson, San Jose, CA) for 5 days and harvested 18 h after [^{3}H]thymidine pulsing.

Table 1
Clinical characteristics of patients divided in group A and group B included in the study.

Patients	Gender	Age	Clinical features	HIES score	Serum IgE levels (kU/l)
1	M	6	Asthma	0	<2000
2	M	6	Asthma	1	<2000
3	M	7	Asthma	1	<2000
4	M	9	Asthma, rhinitis	8	<2000
5	M	11	Asthma	4	<2000
6	M	8	Asthma	1	<2000
7	F	15	Asthma	4	<2000
8	M	6	Asthma, rhinitis	8	<2000
9	M	6	Atopic dermatitis	8	<2000
10	M	10	Asthma	0	<2000
11	M	7	Atopic dermatitis	10	<2000
12	M	5	Atopic dermatitis	10	>2000
13	M	5	Asthma	13	>2000
14	M	7	Asthma	13	>2000
15	M	8	Asthma, rhinitis	13	>2000
16	M	10	Asthma	13	>2000
17	M	15	Asthma	10	>2000
18	M	12	Asthma	13	>2000
19	M	5	Atopic dermatitis	10	>2000
20	M	7	Asthma	10	>2000

2.3. Generation of Th1 cell lines

Th1 cell lines were generated by stimulating PBMC with PHA (8 μg/ml) or, in a few experiments, with PHA + IFN-γ (1000 U/ml, ICN, Biomedical, OH) for 72 h in complete tissue culture medium. These cells usually widely express high affinity IL-12R.

2.4. Analysis of STAT4 activation

PHA-induced blasts were made quiescent by 12 h incubation in RPMI supplemented with 2.5% FCS at RT, and further stimulated with rIL-12 (Genetics Institute, Cambridge, MA) at a concentration of 10–100 U/ml for 10 min. After the appropriate stimuli, $3–5 \times 10^6$ cells were lysed in buffer containing 20 mM Tris, pH 8, 10% glycerol, 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mM sodium orthovanadatum (Na3Vo4), 5 μg/ml leupeptin and 5 μg/ml aprotinin. Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes and then blocked with 5% bovine serum albumin. Immunoblotting was performed by a 2–4 h incubation with anti-STAT4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using chemiluminescence (ECL system, Amersham, Buckinghamshire, England). The low migration supershifted form of STAT4 indicates the presence of the protein in its activated/phosphorylated form [21]. Densitometric analysis was performed to evaluate the overall amount of the protein and the amount of its supershifted form.

2.5. Membrane expression of β1 and β2 chains of IL-12R on T cells

After washing in PBS, cells were incubated for 20 min sequentially with murine anti-β1 or anti-β2 chain (25 μl) of IL-12R (kindly provided by Dr. Jerome Ritz, Dana Farber Cancer Institute, Boston, MA), IgG1 isotype control Ab, 10 μl FITC-conjugated goat anti-mouse IgG Ab (Becton Dickinson, San Jose, CA), and 5 μl anti-CD4 PE Ab (Becton Dickinson, San Jose, CA). After staining, the expression of IL-12Rβ1 and β2 on CD4$^+$ cells was determined with flow cytometer (Becton Dickinson) by gating on the CD4$^+$ population.

2.6. Analysis of IL-12Rβ2 chain RNA expression

Total cellular RNA was prepared using Trizol reagent method (Sigma Chemical Co., St. Louis, MO); 1 μg of total RNA was reverse transcribed into cDNA using Expand™ Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim, Germany). The cDNA was PCR amplified (94 °C, 1 min; 55 °C, 1 min; 72 °C,1 min for 30 cycles) using specific primers for IL-12Rβ2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. Each PCR mixture consisted of 3 μl of cDNA, 1 μl of each primer (concentration from Kathy), 0.2 mM dNTP and 2.5 U of Taq DNA polymerase (Life Technologies Ltd., Paisley, Scotland). These reactions were carried out in a buffer

containing 25 mM $MgCl_2$, 200 mM Tris–HCl and 500 mM KCl. To monitor the amount of RNA, β-actin mRNA expression was used. PCR products were separated in a 1% agarose gel and viewed after ethidium bromide staining.

2.7. Statistical analysis

The significance of differences was evaluated by Wilcoxon rank sum test for unpaired data. All the data were obtained from at least three distinct experiments performed in a 6 months period.

3. Results

3.1. Proliferative responses

Fig. 1A illustrates the proliferative response to CD3 cross-linking (CD3 X-L) performed at optimal antibody concentration, that mimics *in vivo* antigen exposure in patients and controls. Group A patients showed a significantly lower response than controls (mean ± SD: 23,200 ± 6402 *versus* 49,690 ± 4398 cpm in controls, $p < 0.05$). In contrast, patients of group B had a higher proliferative response not significantly different from controls. Similarly, the proliferative response to PHA was lower in group A than in the other groups (mean ± SD: group A, 58,790 ± 11,690 cpm; group B, 106,500 ± 10,800 cpm; controls 93,070 ± 4455 cpm. A *versus* B and A *versus* controls: $p < 0.01$). No difference was found in the proliferative assays with the other stimuli. As depicted in Fig. 1B, the allogeneic response was comparable in the three groups.

3.2. Analysis of STAT4 tyrosine phosphorylation/activation

IL-12/IL-12 receptor signaling plays a crucial role in Th1 induction. To evaluate whether the low response to CD3 X-L associated with a normal allogeneic response was due to an impaired Th1 generation, we next investigated IL-12R signaling by analyzing supershift of the transcription factor STAT4, that promptly occurs after receptor triggering by its own cytokine and indicates protein tyrosine phosphorylation of the molecule [21]. Fig. 2A shows a representative experiment out of six performed indicating that in controls rIL-12 stimulation for 10 min of PHA-induced cell lines induces STAT4 supershift, due to the appearance of a slower migrating form representing the phosphorylated molecule. By contrast, in all patients of group A there was no supershift of STAT4, and the molecule appeared as a single form of 84 kDa. Fig. 2B illustrates the densitometric analysis representing the overall amount of STAT4 and the amount of its supershifted form. The protein was expressed in patients and controls in a comparable amount. IL-12 stimulation induced the supershift only in control cells and not in patient cells. Fig. 3A shows that IL-12 stimulation of cell

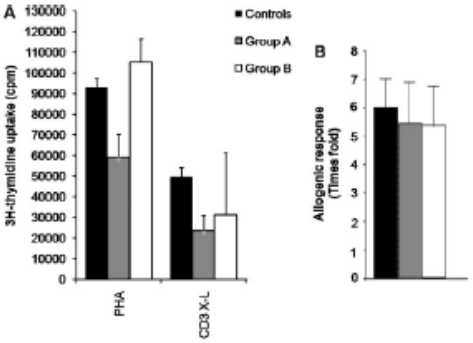

Fig. 1. Proliferative responses in patients and controls. Patients were divided on the basis of IgE levels (group A, $n = 10$, IgE >2000 kU/l; group B, $n = 10$, IgE value between the age specific mean ± 2 SD and 2000 kU/l; controls, $n = 20$). (A) Proliferative response to PHA (8 μg/ml) and CD3 cross-linking (CD3 X-L), performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody. Each column represents the mean value ± SD. (B) Proliferative response to allogeneic stimuli. Results are expressed as the mean value ± SD and indicate the times fold increase over the background.

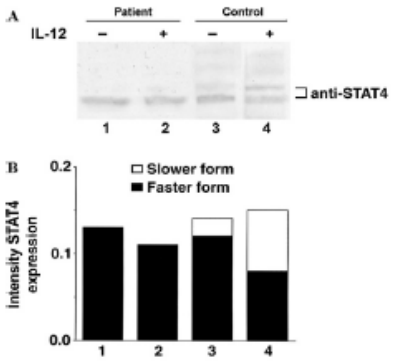

Fig. 2. Analysis by immunoblot of STAT4 protein in controls and patients with very high IgE levels (>2000 kU/l). (A) Representative experiment, out of 6, showing that rIL-12 stimulation induces in controls the appearance of a slow migrating phosphorylated form of the protein, whereas in patients only the 84 kDa protein is evident. PBMC from a patient (lanes 1 and 2) or control (lanes 3 and 4) were incubated with PHA for 72 h, and then further stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein, the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

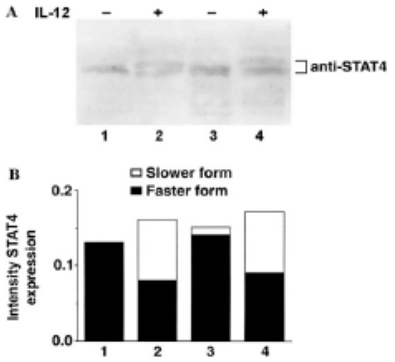

Fig. 3. Analysis by immunoblot of STAT4 protein in controls and patients with IgE values between the age specific mean ± 2 SD and 2000 kU/l. (A) Representative experiment, out of 3, showing that rIL-12 stimulation induces both in control and patient the appearance of a slower form of STAT4. PBMC were processed as indicated in Fig. 2 and Section 2. Lanes 1 and 2, patient; lanes 3 and 4, control. Cells were stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein; the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

lines obtained from patients of group B, induced the appearance of the slower supershifted form of STAT4 both in controls and patients to a similar extent.

3.3. IL-12R expression on T cells

The high affinity IL-12 receptor consists of β1 and β2 chains, the latter being up-regulated during cell activation and selectively expressed on Th1 cells. To determine whether the failure of STAT4 phosphorylation was due to decreased expression of IL-12 receptor, we analyzed the surface expression of IL-12R in T cell lines induced in the presence of PHA. The expression of both β1 and β2 chains was lower in group A than in the other groups. IL-12Rβ1 values, expressed as mean percentage of positively stained cells ± SD, were as follows: group A, 35.96 ± 7.3%; group B, 53.8 ± 6.6%; controls, 51.7 ± 6.1%. Similarly, a lower up-regulation of β2 chain in group A was observed as depicted in Fig. 4A. Mean percentage values ± SD of IL-12Rβ2 expression were 16.5 ± 3.0% in group A; 28.8 ± 3.7% in group B; 28.9 ± 1.6% in controls (*A versus* B and controls: $p < 0.05$). The mean fluorescence intensity was lower in the group A than in the other groups (Fig. 4B), differently from β1 whose intensity was comparable in the three groups (data not shown).

3.4. IL-12Rβ2 mRNA expression

We next analyzed the mRNA expression of IL-12Rβ2 chain in group A, where no STAT4 tyrosine phosphorylation was observed. The expression of the IL-12Rβ2 transcript in all experiment performed was different between patients and controls. In three experiments there was no induction at all of β2 transcript after of 18, 36 and 48 h PHA stimulation, as illustrated in a representative experiment in Fig. 5. Furthermore, no effect of IFN-γ was noted. These data were confirmed by five distinct experiments. In two cases there was mRNA expression, but in one case it was delayed appearing only after 48 h PHA stimulation, even though it

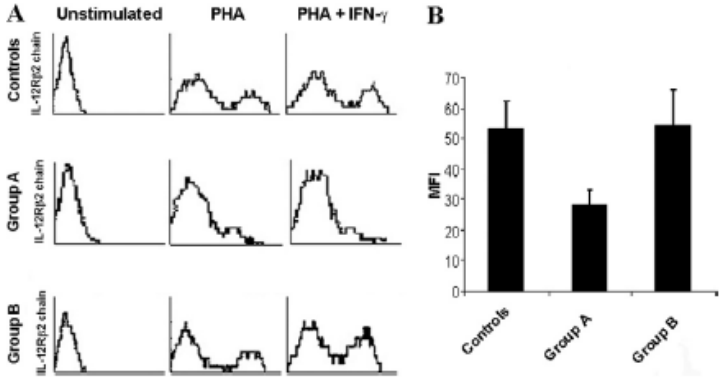

Fig. 4. Membrane expression of β2 chain of IL-12R on T cells. IL-12Rβ2 membrane expression on resting or T-cell blasts, induced by stimulation with PHA for 72 h in the absence or presence of IFN-γ, in controls and patients divided in two groups on the basis of IgE levels as indicated in Section 2. Dual colour fluorescence using FITC-conjugated anti-β2 and PE-conjugated anti-CD4 was performed. (A) Shows a representative experiment indicating the lower up-regulation of β2 chain in group A. The mean fluorescence intensity in the three groups is shown in (B). Each column represents the mean value ± SD. The intensity was lower in the patients of group A than in the other groups.

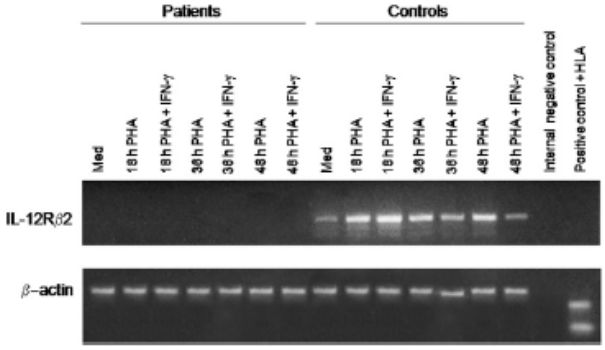

Fig. 5. mRNA expression of IL-12Rβ2 chain in controls and group A patients (IgE levels: >2000 kU/l). Representative experiment showing that in controls, β2 chain mRNA expression increased after 18 h of PHA stimulation, lanes 1 and 8: freshly isolated PBMC. T-cell blasts were generated by 18, 36 and 48 h of PHA stimulation. IFN-γ upregulated β2 chain mRNA expression after short term PHA stimulation, but it was ineffective during longer stimulations. In group A there was no induction at all of β2 transcript. Furthermore, no effect of IFN-γ was noted.

was also slightly appreciable after 36 h stimulation in the presence of IFN-γ. In the other case a faint signal was appreciable after 18 h of PHA stimulation, but it rapidly disappeared.

4. Discussion

In this study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction. In particular, defective supershift of the STAT4 molecule following rIL-12 stimulation of T-cell blasts was documented. Supershift of this molecule indicates its phosphorylation [21]. This finding was associated with a T-lymphocyte functional derangement characterized by low proliferative response to stimulations via TCR/CD3 complex, but with a preserved allogeneic response. The discrepancy between mitogenic and allogeneic stimuli in inducing cell proliferation has already been documented in mice in which the gene coding for 40 kDa subunit of IL-12 has been disrupted [17]. These mice are not able to generate most of the Th1 responses, including IFN-γ production and delayed type hypersensitivity response *in vivo*, but cytolytic response elicited by allogeneic stimuli was preserved, thus suggesting that the allogeneic response is dependent on a wider array of cytokines influences. Further evidence on the role of the IL-12/IL-12R signaling apparatus on the induction of Th1 responses comes from the functional studies on mice lacking STAT4 molecule, that represents a central signaling protein involved in IL-12R signaling [22]. Although there is evidence suggesting that the development of Th1 type responses may also take place in a STAT4 independent fashion [23,24], the STAT4 knock-out experimental model underlines the importance of the integrity of the IL-12/IL-12R signaling for the generation of a proper Th1 type response. Again, $STAT4^{-/-}$ mice exhibit a propensity to generate Th2 type cells [22]. It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut-off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by Hyper-IgE Syndrome (HIES). However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed.

The link between viral and bacterial infections and the pathogenesis of allergic asthma has represented for years an appealing area of clinical investigation, which is currently expanding in parallel with the worldwide increase of childhood asthma prevalence [25]. Longitudinal studies indicate that respiratory tract infections may predispose children to asthma [26]. Persistent wheezing seems to be related to increased IgE levels and eosinophils at the time of the first respiratory infection, thus suggesting that infections may trigger asthma attacks in already predisposed subjects [27,28]. However, in contrast to this, it has been shown that early infections may protect against the subsequent development of an atopic phenotype [29]. This hypothesis is also supported by the recently documented inhibitory effect on Th2 cell functions of Th1-released proinflammatory cytokines [30]. Public health measures, as hygiene programs to reduce foodstuffs contamination, active immunization programs, a better pharmacological control of infections, may certainly have contributed in limiting the immune system challenge by infectious agents in early childhood, even though hygiene hypothesis should be revisited in the light of recent data on the role of Toll like receptors and regulatory mechanisms [31]. However, in our study it should be noted that there were not striking differences between the three groups of subjects with regards to vaccination program, social habits and the number or severity of infections in the clinical history that preceded the appearance of allergic disorders.

However, it should be noted that abnormalities of IL-12 signaling pathway is not sufficient *per se* to cause an allergic disease in children, in that the prevalence of asthma, eczema and rhinoconjunctivitis is similar in patients with or without genetic alteration of IFN-γ or IL-12Rβ1 [32]. Our data could imply a link between infections and allergy in children, even though this matter is still under debate and no conclusive demonstration is available [31]. In the light of this consideration, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help ameliorate the overall management of these patients. In fact, there is evidence that patients with severe forms of allergic manifestations are more susceptible to respiratory infections, and vice versa infections may trigger acute episodes of asthma [25]. A defective induction of a Th1 response in patients with very high IgE levels may lead to a higher risk of infections, thus worsening the overall outcome.

Overall, our results indicate that children with very high serum IgE levels have functional and biochemical signs of an altered IL-12/IL-12 receptor signaling network.

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§ 3.2 Conclusive remarks

In this study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction. In particular, defective supershift of the STAT4 molecule following rIL-12 stimulation of T-cell blasts was documented. It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut-off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by Hyper-IgE Syndrome (HIES). However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features [110, 111]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed. Thus, also in this part of the thesis, there is a description of a novel aspects of a well known phenotype associated with immune dysfunction. In particular, in this case we characterized an unappreciated relationship between an impairment of Th1 induction due to alteration of IL-12R signalling and high levels of serum IgE but in the absence of HIES. Moreover, since abnormalities of IL-12 signaling pathway is not sufficient *per se* to cause an allergic disease in children, in that the prevalence of asthma, eczema and rhinoconjunctivitis is similar in patients with or without genetic alteration of IFN-γ or IL-12Rβ1 [112], these data could imply a link between infections and allergy in children, even though this matter is still under debate and no conclusive demonstration is available [113]. In the light of this consideration, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help to ameliorate the overall management of these patients.

Technologies

Nude/SCID and DGS patients

The patients included in the first part of the study were children affected by rare primary immunodeficiencies including Nude/SCID and DGS mostly identified at the Unit of Immunology of the Department of Pediatrics at the "Federico II" University of Naples where they were followed. Moreover, other patients were identified through clinical and molecular diagnostic criteria according to the European Society of Immunodeficiencies. DGS patients were selected for the presence of one of the following presenting signs:

- deletion of the Cr. 22q11.2, studied by FISH analysis
- thymic aplasia, agenesis and hypoplasia

and for the concomitant presence of the following typical DGS features, such as:

- congenital cardiopathy
- hypoparathyroidism/hypocalcemy
- dysmorphism
- microcephaly

Moreover, Nude/SCID patients were selected for the presence of:

- FOXN1 gene mutation
- thymic agenesis
- alopecia
- nail dystrophy

DNA analysis

After a written informed consent was obtained from parents, genomic DNA was extracted by standard procedures from villous and whole blood and processed. *FOXN1* DNA analysis was performed according to a

polymerase chain reaction (PCR) assay previously described.[48] Briefly, a PCR fragment containing exon 4 of the FOXN1 gene (formerly named exon 5) was amplified using the primers exon 5F: 5#-CTTCTGGAGCGCAGGTTGTC-3# and exon 5R: 5#-TAAATGAAGCTCCCTCTGGC-3#. The PCR product was sequenced using an ABI prism 310 Genetic Analyzer (Applied Byosystems Inc.).

Microsatellite analysis for maternal contamination CBMC

To test maternal contamination, multiplex-PCR of 15 highly polymorphic autosomal short tandem repeat loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA) was performed on DNA samples from cord blood using AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems).

Cell staining and proliferation assay of Nude/SCID and DGS lymphocytes

After written consent, cord blood mononuclear cells (CBMCs) and peripheral blood mononuclear cells (PBMCs) were isolated from Nude/SCID fetus and control human fetus, matched for the same gestational ageand from other Nude/SCID patients and DGS patients, respectively, by density gradient centrifugation over Ficoll-Hypaque (Biochrom). Cell suspensions were prepared in RPMI 1640 medium. CBMC and PBMC were cultured in triplicate in 96-well U-bottomed microtiter plates and after stimulation with 8 µg/mL phytohemagglutinin (PHA), with 10 µg/mL PWM, 8.25 µg/mL Con-A and 1 ng/mL and 0.1 ng/mL anti-CD3 mAb, previously precoated on tissue culture plates for cross-linking, cell proliferation was determined by incorporation of tritiated thymidine for 72 hours of culture. In the

experiment performed in Hyper IgE study also the Phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) were evaluated.

CBMC were stained with the appropriate antibodies: CD45, CD34, CD19, CD4, CD8, CD62L (Immunotech Coulter) CD3, CD16, CD56, CD7, CD45RA, (BD Pharmingen) on ice, washed and analyzed using a FACSCanto II flowcytometer Becton Dickinson. Data were analyzed using FACSDiva software.

MRI imaging of the fetal brain

Brain MRI study was performed at 3 Tesla (Magnetom TRIO, Siemens, Germany) using a 3D low-angle, gradient-echo sequence (TR/TE 572/3.7 msec, FA 9°, FOV150mm, acquisition matrix 144x256, slice thickness 600mm, using a quadrature volumetric coil) providing a T1w volume which was resliced along axial, coronal and sagittal planes. Images were processed using GIMP (http://www.gimp.org) to remove paraformaldehyde background.

Subjects included in Hyper IgE study

Twenty patients with elevated IgE levels and history of allergy were enrolled into the study. Sixteen patients were affected by asthma, 3 of them also by rhinitis, and 4 had a history of atopic dermatitis. The patients divided in two subgroups on the basis of IgE levels: group A consisted of 10 patients, 10 males, range of age 5-15 yr, with very high serum IgE levels (> 2000 kU/l, range 2152-5000 kU/l); group B consisted of 10 patients, 9 males, range of age 6-15 yr, with high serum IgE levels (IgE value between the age specific mean ± 2SD and 2000 kU/l, range 93-1152 kU/l). Twenty healthy controls, 16 males range of age 6-15 yr (IgE range 85-100 kU/l), were also studied. Informed consent was obtained when required. No difference was found between group A and B in either the number per year or the severity of

allergic manifestations. The Hyper-IgE syndrome (HIES) was excluded by the absence of typical clinical and immunological features [110]. In particular, no eczema and recurrent skin infections, facial, skeletal and dentition anomalies were observed. Other conditions accompained by elevated serum IgE concentration, including AIDS, helminths and parasitic infections were also excluded by clinical and laboratoristic features. The study has been approved by the applicable Institutional Review Board.

Generation of Th1 cell lines

Th1 cell lines were generated by stimulating PBMC with PHA (8 μg/ml) or, in a few experiments, with PHA + IFN-γ (1000 U/ml, ICN, Biomedical, OH) for 72 hours in complete tissue culture medium. These cells usually widely express high affinity IL-12R.

Analysis of STAT4 activation

PHA-induced blasts were made quiescent by 12 hours incubation in RPMI supplemented with 2.5% FCS at RT, and further stimulated with rIL-12 (Genetics Institute, Cambridge, MA) at a concentration of 10-100 U/ml for 10 minutes. After the appropriate stimuli, 3-5 x 10^6 cells were lysed in buffer containing 20 mM Tris, pH 8, 10% glycerol, 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mM sodium orthovanadatum (Na3Vo4), 5 μg/ml leupeptin and 5 μg/ml aprotinin. Proteins were resolved by 10% SDS- polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes and then blocked with 5% bovine serum albumin. Immunoblotting was performed by a 2-4 hours incubation with anti-STAT4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using chemiluminescence (ECL system, Amersham, Buckinghamshire, England). The low migration supershifted form of STAT4 indicates the presence of the

protein in its activated/phosphorylated form [114]. Densitometric analysis was performed to evaluate the overall amount of the protein and the amount of its supershifted form.

Membrane expression of β1 and β2 chains of IL-12R on T cells

After washing in PBS, cells were incubated for 20 minutes sequentially with murine anti-β1 or anti-β2 chain (25 μl) of IL-12R (kindly provided by Dr. Jerome Ritz, Dana Farber Cancer Institute, Boston, MA), IgG1 isotype control Ab, 10 μl FITC-conjugated goat anti-mouse IgG Ab (Becton Dickinson, San Jose, CA), and 5 μl anti-CD4 PE Ab (Becton Dickinson, San Jose, CA). After staining, the expression of IL-12R-β1 and β2 on $CD4^+$ cells was determined with flow cytometer (Becton Dickinson) by gating on the $CD4^+$ population.

Analysis of IL-12R-β2 chain RNA expression

Total cellular RNA was prepared using Trizol reagent method (Sigma Chemical Co., St. Louis, MO); 1 μg of total RNA was reverse transcribed into cDNA using ExpandTM Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim, Germany). The cDNA was PCR amplified (94°C, 1 minute; 55°C, 1 minute; 72°C,1 minute for 30 cycles) using specific primers for IL-12R-β2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. Each PCR mixture consisted of 3 μl of cDNA, 1 μl of each primer (concentration from Kathy), 0.2 mM dNTP and 2.5 U of Taq DNA polymerase (Life Technologies LTD, Paisley, Scotland). These reactions were carried out in a buffer containing 25 mM MgCl2, 200 mM Tris-HCl and 500 mM KCl. To monitor the amount of RNA, β-actin

mRNA expression was used. PCR products were separated in a 1% agarose gel and viewed after ethidium bromide staining.

A-T Patients

Blood samples were obtained from six consecutive patients (3 males), of 5, 6, 14, 15, 27 and 29 years of age, respectively (mean age 16.3 years, range 5-29 years), affected with A-T. The patients, upon written consent, received a cycle of oral betamethasone at the dosage of 0.1 mg/kg/day for 10 days divided every 12 hours. No antioxidants, as N-acetyl cysteine, vitamin E or alphalipoic acid, were given to the patients during the two years preceding the trial. The clinical evaluations were carried out before therapy (T0), 48 h from the beginning (T48), at the end of the therapy (T10d) and, eventually, 7 days from the withdrawal (T7 off therapy). The neurological evaluation was performed through the Scale for the Assessment and Rating of Ataxia (SARA).

Determination of lipid peroxidation

Cells ($0.8x10^5$) were lysed by freeze and thaw in 10 mM TRIS pH 7.5 and the lipid peroxides were assessed with Cayman Chemical Company assay kit (Ann Arbor, Michigan, USA), according to the manufacturer's instruction. This kit measures malonaldehyde (MDA) and 4-hydroxyalkenals that result from peroxidation of polyunsaturated fatty acids. The samples were normalized for cell number.

Evaluation of intracellular glutathione

The cells were lysed by freeze and thaw. Cellular levels of GSH were determined using DIGT-250 GSH colorimetric assay kit (BioAssay Systems, California, USA). Cells ($0.8x10^5$) were treated for 48 h with various agents and collected by centrifugation at 1300 g for 10 min at 4°C. All the samples

were analyzed following manufacturer's instructions. Optical density (OD)-values were read at 412 nm. The samples were normalized for cell number.

Measurement of intracellular ROS

The cellular ROS accumulation was measured using the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) method. DCFH-DA is a non-fluorescent compound, and it can be enzymatically converted to highly fluorescent compound, DCF, in the presence of ROS. After and before exposure to betamethasone, $1x10^6$ cells were treated with 5 μM DCFH-DA at 37°C for 20 min, at 5% CO2, washed with PBS, collected and analyzed immediately on a FACScan flow cytometer (Becton Dickinson, New Jersey, USA) equipped with an argon laser emitting at 488 nm. Cell Quest software (Becton Dickinson, New Jersey, USA) was used to acquire and evaluate all the events.

Summary

In the last thirty years of the 20th century, a formidable numbers of scientific discoveries in the field of PIDs were made. Many scientific papers have been published on the molecular and cellular basis of the immune response and on the mechanisms involved in the correct ontogeny of immune system components. Today we know the genetic and molecular basis of the principal mechanisms involved in the immune response, thus it's possible to classify immune diseases in different way, by different points of view. In this thesis, during the three years of my PhD program, I tried to group the congenital immunodeficiencies on the basis of the altered immune function and on the basis of the cell type where the mutated genes are expressed in order to perform a novel characterization of such diseases through the combination of clinical, cellular, functional and molecular approaches.

In particular, my research work was focused on the study at both molecular and clinical levels of the Nude/SCID phenotype as the real human model of athymia in comparison with the DiGeorge syndrome which was erroneously considered for long time the human counterpart of the Nude murine model.

Moreover, in this context, I also participated to better define the functional role of FOXN1 transcription factor in the development of the pleiotropic aspects of the Nude/SCID syndrome. From this study, FOXN1 emerged as a cofactor in the development and differentiation of some structures in the central nervous system. Of note, these immunodeficiencies are due to mutated genes expressed in non hematopoietic cells.

In addition, I participated to the study of patients affected with Ataxia Telangiectasia (A-T) whose causing gene is not selectively expressed in the hematopoietic compartment and related to cerebellar functions. In this context I contributed to evaluate whether the beneficial effect of

betamethasone therapy in (A-T) patients could be mediated by interference in ROS generation/neutralization process. Our research led to conclude that betamethasone response in A-T patients is inversely correlated to cerebellar atrophy and directly to antioxidative capacity.

During my PhD course I also participate to the study of some immunodeficiencies due to alterations of genes expressed in hematopoietic cells giving a contribution to the description of the mechanism altered in the case of high serum level of IgE. The data obtained in this context revealed the alteration of IL-12R signaling in a group of patients with high levels of serum IgE but without Hyper IgE Syndrome.

Overall, all my studies were designed in order to better understand the mechanisms involved in the pathogenesis of such immune diseases. The results of this thesis by adding novel information in this field could be useful both in the clinical practice and in the basic research of immune disorders by revealing unknown aspects of these diseases.

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Appendix I – Curriculum Vitae

PERSONAL INFORMATION	
Name	**FUSCO, Anna**
Address	**Via Lombardia, 43 - 84096 Montecorvino Rovella (SA)- Italy**
Telephone	+39-089-863036/ +39-329-4224334
Fax	
E-mail	afusco3@inwind.it
Nationality	Italian
Date of birth	30, October, 1982
EDUCATION AND TRAINING	
• Dates	From September 2010 - to October 2010
• Name and type of organisation providing education and training	Genomic Stability Unit of Clare Hall Laboratories, Cancer Research UK – Blanche Lane, South Mimms, EN6 3LD, London – United Kingdom
• Principal subjects/occupational skills covered	Fellowship to perform a fluorimetric assay to evaluate the enzymatic activity of G6PD in patients affected with Ataxia Teleangiectasia before and after bethametasone treatment.
• Date	2008
• Name and type of organisation providing education and training	"Federico II" University of Naples, Naples - Italy.
• Title of qualification awarded	Licence to practice as a Biologist.
• Dates	From 2007 - to 2010
• Name and type of organisation providing education and training	Unit of immunology, Department of Pediatric at the "Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
• Principal subjects/occupational skills covered	Ph.D. student at the Doctoral Course in "Riproduzione, Sviluppo e Accrescimento dell'uomo" with a research project focused on the study of human models of athymia (Nude/SCID syndrome and DiGeorge Syndrome) through the characterization of peripheral and tissue lymphocytes in order to identify an alternative extrathymic sites of T-cell ontogeny in the absence of the thymus.
• Title of qualification awarded	PhD degree will be awarded in December 2010
• Level in national classification	ISCED 7
• Dates	From 2005 - to 2007
• Name and type of organisation	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.

providing education and training	
• Principal subjects/occupational skills covered	Internship at the Unit of Immunology, Department of Pediatrics of the "Federico II" University of Naples, Naples, Italy, focused on the study of a previously unappreciated relationship between common gamma chain and growth hormone receptor and on the molecular characterization of genes involved in apoptotic pathways in patients with cluster of autoimmune diseases
• Title of qualification awarded	Bachelor in Medical Biotechnology (II level degree) at the "Federico II" University of Naples, with a thesis entitled: “Valutazione dell’effetto del silenziamento molecolare della catena sul signaling del recettore del GH in linee cellulari B”. Vote: 110/110
• Level in national classification	ISCED 6
• Dates	From 2003 - to 2004
• Name and type of organisation providing education and training	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
• Principal subjects/occupational skills covered	Internship at the Department of Medical Biochemistry and Biotechnology (DBBM) of the "Federico II" University of Naples, focused on the study of HLA aplotypes by different technical approaches.
• Title of qualification awarded	Bachelor in Biotechnology for Healthcare (I level degree) at the "Federico II" University of Naples with a thesis entitled: “La tipizzazione HLA: metodi a confronto”. Vote: 104/110.
• Level in national classification	ISCED 6

PERSONAL SKILLS AND COMPETENCES

Acquired in the course of life and career but not necessarily covered by formal certificates and diplomas.

MOTHER TONGUE	**ITALIAN**
OTHER LANGUAGES	
	ENGLISH
• Reading skills	Good B1
• Writing skills	Good C2
• Verbal skills	Basic B1
SOCIAL SKILLS AND COMPETENCES *Living and working with other people, in multicultural*	Predisposition to work in a team of 5-6 persorns by collaborating each other in a friendly manner developed during my PhD period. Capacity to interact with other collegues also in multicultural environmnets developed during my fellowship at the clare hall laboratories in London.

environments, in positions where communication is important and situations where teamwork is essential (for example culture and sports), etc.

ORGANIZATIONAL SKILLS AND COMPETENCES

Coordination and administration of people, projects and budgets; at work, in voluntary work (for example culture and sports) and at home, etc.

Capacity to design a scientific project including the economic budget (See the attached list of application to grant proposals).

Capacity to administrate small budgets for the daily work in a small lab.

Capacity to coordinate students in their practice in lab also by follow them in the preparation of the thesis.

TECHNICAL SKILLS AND COMPETENCES

With computers, specific kinds of equipment, machinery, etc.

- use of edit view 1.0 and inherit autoassembler 1.4.0 to molecular analysis of genes.
- use of oligo4 (for mac) to design primers.
- knowledge of entrezgene, genecards, embl nucleotide sequence database and of the UCSC genome browser.
- ability to perform a proliferative assay through the evaluation of thymidine incorporation by lymphocytes pre-stimulated with mitogens.

The above cited competences were acquired during my PhD at the dept of Pediatrics where I also participated to clinical practice by diagnosting some immune disorders.

- use of endnote 7.0 to format and add references to a manuscript.
- use of Word, graphical softwares such as PowerPoint, Publisher, Photoshop for Mac and Win, statitistical softwares such as Excel e GraphPad Prism and softwares to elaborate images such as ImageJ 1.42.

The above cited competences were acquired during my PhD at the dept of Pediatrics where I also participated to the preparation of an entire scientific paper also by creating imagines and graphics and to the preparation of lessons, seminars and posters for congress and meetings (see the attached list of scientific production)

OTHER SKILLS AND COMPETENCES

Competences not mentioned above.

SCIENTIFIC SKILLS

- DNA and RNA extractions
- PCR and Sequencing analysis
- Transfection and RNA interference
- Cell cultures
- Western blot
- Immunofluorescence

ADDITIONAL INFORMATION

SCIENTIFIC INTERESTS

Major fields of my scientific interests are as follows:

- Regulatory mechanisms governing lymphocyte cell proliferation, activation and cell death. In particular, the current interest is to characterize novel aspects in immunodeficiencies, with a particular regard to Severe Combined immunodeficiency (SCID);
- Molecular analysis of genes whose mutations are responsible for certain immunodeficiencies, such as Perforin, Caspase 8 and FOXN1.
- T-cell ontogeny process in human. In particular, the focus is to identify possible extrathymic sites of T-cell differentiation by comparing lymphocytic phenotypes from Nude/SCID and DiGeorge patients that represent two different models of human athymia.
- Studies of previously unappreciated relationships between receptor signaling systems in the pathogenesis of SCIDs;
- Signal transduction in physiology and human diseases affecting the immune system.
- Primary Immunodeficiencies: definition of novel therapeutical strategies for the treatment of Ataxia-Teleangiectasia. In particular, I participated in the activity of Prof. Pignata group during a clinical trial based on the use of bethametasone to improve neurological function in the affected patients. In keeping with this, I attended the Genomic Stability Unit of Clare Hall Laboratories at the Cancer Research UK, in London to perform a fluorimetric assay to evaluate the enzymatic activity of G6PD in patients affected with Ataxia Teleangiectasia before and after bethametasone treatment.

SCIENTIFIC PRODUCTION

(Dr. Anna Fusco)

LIST OF PUBLICATIONS

1. T. Broccoletti**,** E. Cirillo, G. Aloj, I .Russo, **A. Fusco**, C. Pignata. I quattro paradossi delle Immunodeficienze. Rivista Italiana di Allergologia e Immunologia Pediatrica • 03/2008 • 10-21

2. Russo I., Cosentino C., Del Giudice E., Broccoletti T., Amorosi S., Cirillo E., Aloj G., **Fusco A**., Costanzo V., Pignata C. In Ataxia-Teleangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. Eur J Neurol 16: 755-759, 2009.

3. Pignata C., **Fusco A**. (2008). Le immunodeficienze congenite: il modello Nude/SCID. In: Bona G. Minerva pediatrica. (vol. 60, pp. 900-901). Minerva pediatrica.

4. Pignata C., **Fusco A**. and Amorosi S. Human clinical phenotype associated with FOXN1 mutations. From the book Forkhead Transcription Factors: Vital Elements in Biology and Medicine co-published by Landes Bioscience and Springer. Vol. 665: 195-206, 2009

5. Pignata C., **Fusco A**., Amorosi S., Vigliano I., Genovese V., Aloj G. and Valentino L. La sindrome Nude/SCID: dal modello murino al fenotipo umano. Rivista Italiana di Genetica e Immunologia Pediatrica. Anno I, Numero 2, giugno 2009.

6. Pignata C., **Fusco A**. and Amorosi S. Human clinical phenotype associated with FOXN1 mutations. Adv Exp Med Biol. 2009;665:195-206.

7. Calcagno G., Ursini M.V., Castaldo G., **Fusco A**., Martinelli P., Pignata C. and Salvatore F. Diagnosi prenatale della sindrome Nude/SCID. Pediatric Reports 1:s1: 29, 2009.

8. **Fusco A**, Vigliano I, Palamaro L, Cirillo E, Aloj G, Piscopo G, Giardino G, Pignata C. Altered signaling through IL-12 receptor in children with very high serum IgE levels. Cell Immunol. 265:74-79, 2010.

9. Stefania Amorosi, Ilaria Vigliano, Ennio Del Giudice, Luigi Panico, Giuseppe M. Maruotti, **Anna Fusco**, Mario Quarantelli, Carla Ciccone, Matilde V. Ursini, Pasquale Martinelli, Claudio Pignata. Brain alteration in a Nude/SCID fetus carrying *FOXN1* homozygous mutation. 2010 (In Press on J Neurol Sciences)

MEETING ABSTRACTS AND COMMUNICATIONS

1. Amorosi S., Guarino V., **Fusco A**., Vigliano I., Gorrese M., Del Vecchio L., Ambrosio L., Pignata C. Comparazione del blocco ontogenetico T nei due modelli umani di atimia Nude/SCID e DiGeorge e allestimento di uno "scaffold" tridimensionale per la generazione in vitro di cellule T da precursori ematopoietici in assenza di timo. Giornata di Ricerca del Dipartimento di Pediatria. "Federico II" University, Department of Pediatrics. Naples April 17, 2009. (Oral presentation)

2. **Fusco A**., Amorosi S., Vigliano I., Vitiello L., Racioppi L., Gorrese M., Del Vecchio L., Pignata C. La mutazione del gene FOXN1 associata al fenotipo Nude/SCID previene completamente il differenziamento dei linfociti CD4, ma non dei CD8. Giornata di Ricerca del Dipartimento di Pediatria. "Federico II"

University, Department of Pediatrics. Naples April 17, 2009. (Oral presentation)

3. Amorosi S., **Fusco A**., Vigliano I., Gorrese M., Del Vecchio L., Pignata C. Characterization of the T-cell ontogeny defect in the human athymic models of the Nude/SCID and DiGeorge syndromes. Day of Immunology "Mucosa associated immune responses: between tolerance and inflammation". Avellino April 29, 2009. (Selected oral presentation)

4. **Fusco A**, Amorosi S, Gorrese M, Vigliano I, Vitiello L, Racioppi L, Del Vecchio L and Pignata C. La mutazione del gene FOXN1 associata al fenotipo Nude/SCID previene completamente il differenziamento dei linfociti CD4, ma non dei CD8. "Federico II" University, Department of Pediatrics. Naples February 18, 2010. (Oral presentation)

5. **Anna Fusco**, Marisa Gorrese, Giuseppina Aloj, Ilaria Vigliano, Loredana Palamaro, Emilia Cirillo, Giuliana Giardino, Luigi Del Vecchio and Claudio Pignata. Caratterizzazione del difetto di ontogenesi T nei due modelli umani di atimia, la sindrome Nude/SCID e la sindrome di DiGeorge. 12° SIAIP National Congress. Bari April 14-17, 2010. (Poster presentation)

6. Emilia Cirillo, **Anna Fusco**, Vera Gallo, Filomena Maio, Ilaria Vigliano, Giuliana Giardino, Leopoldo Valentino, Giuseppina Aloj and Claudio Pignata. Alterazioni gastrointestinali in pazienti affetti dalla Sindrome di DiGeorge. 12° SIAIP National Congress. Bari April 14-17, 2010. (Poster presentation)

7. G. Giardino, G. Aloj, F. Maio, V. Gallo, L. Valentino, E. Cirillo, **A. Fusco** and C. Pignata. Comparazione fenotipica intergenerazionale in pazienti affetti da cluster di malattie autoimmuni (CMA). 12° SIAIP National Congress. Bari April 14-17, 2010. (Poster presentation)

8. Ilaria Vigliano, Loredana Palamaro, Laura Vitiello, **Anna Fusco**, Emillia Cirillo, Giuseppina Aloj, Vera Gallo and Claudio Pignata. Correlazione diretta tra livelli di espressione di IL-2Rγ e proliferazione cellulare spontanea in linee cellulari maligne ematopoietiche. 12° SIAIP National Congress. Bari April 14-17, 2010. (Poster presentation)

9. **A. Fusco**, L. Panico, G. Troncone, S. Amorosi, I. Vigliano, C. Pignata. Identification of extrathymic foci of lymphopoiesis in an athymic Nude/SCID human fetus. Day of Immunology "Autoimmunity: from basic immunology to clinics". Naples April 29, 2010. (Oral presentation)

10. I. Vigliano, L. Palamaro, L. Vitiello, S. Amorosi, **A. Fusco** and C. Pignata. Gamma chain expression levels influence spontaneous cell proliferation in different malignant hematopoietic cell lines. Day of Immunology "Autoimmunity: from basic immunology to clinics". Naples April 29, 2010. (Oral presentation)

11. **A. Fusco**, L. Panico, M. Gorrese, I. Vigliano, L. Palamaro, L. Del Vecchio, C. Pignata *Prenatal T-cell ontogeny in human Nude/SCID fetus and extrathymic lymphopoiesis* XIVth meeting of the European Society for Immunodeficiencies. Instanbul, Turkey. 6-9 October 2010 (Poster presentation)

12. I. Vigliano, G. Bianchino, L. Palamaro, V. Grieco, L. Vitiello, **A. Fusco**, E. Cirillo, M. Salvatore, C. Pignata *Effects of gamma chain on cyclins expression and cell cycle progression in malignant hematopoietic cell lines* XIVth meeting of the European Society for Immunodeficiencies. Instanbul, Turkey. 6-9 October 2010 (Poster presentation)

13. E. Cirillo, **A. Fusco**, I. Vigliano, L. Palamaro, G. Aloj, G. Giardino, V. Gallo, F. Maio, L. Valentino , E. Cosentini, and C. Pignata. *Severe combined*

immunodeficiency phenocopy associated with an inhibitory autoreactive immunoglobulin XIVth meeting of the European Society for Immunodeficiencies. Instanbul, Turkey. 6-9 October 2010 (Poster presentation)

AWARDS

1. Amorosi S., Gorrese M., **Fusco A**., Vitiello L., Panico L., Vigliano I., Ursini M. V, Racioppi L., Del Vecchio L., Pignata C. FOXN1 mutation abrogates pre-natal T-cell development in humans. First CIS North American Primary Immune Deficiency National Conference. Philadelphia May 20-23, 2010. (Poster presentation)

2. **Fusco A**., Panico L., Troncone G., Amorosi S., Vigliano I., Valentino L., Pignata C. Identification of extrathymic foci of lymphopoiesis in an athymic Nude/SCID human fetus. First CIS North American Primary Immune Deficiency National Conference. Philadelphia May 20-23, 2010. (Poster presentation)

3. Vigliano I., Amorosi S., **Fusco A**., Vitiello L., Palamaro L., Maio F:, Gallo V., Pignata C. Gamma chain expression level influences spontaneous cell proliferation in different malignant hematopoietic cell lines. First CIS North American Primary Immune Deficiency National Conference. Philadelphia May 20-23, 2010. (Poster presentation)

MANUSCRIPT IN PREPARATION

- Anna Fusco, Luigi Panico, Marisa Gorrese, Gabriella Bianchino, Laura Vitiello, Ilaria Vigliano, Loredana Palamaro, Luigi Del Vecchio, and Claudio

Pignata. Prenatal extrathymic lymphopoiesis in human athymic Nude/SCID due to *FOXN1* alteration. (For J Exp Med evaluation)

Appendix II – Grant Proposals

During my PhD course, within my research group, coordinated by Professor Claudio Pignata, I have participated to the following Applications for Grant:

- Call for Firb Application 2008 (MIUR) - Programma "Futuro in Ricerca", with a project entitled: **"**Characterization of the T-cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes";
- "TELETHON GRANT PROPOSALS-Call for Applications 2009" with a project entitled: "Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes";
- Call for Application in Malattie Rare 2009 (Ministero della Salute) with a project entitled: "Study of the T-cell ontogeny defect in the murine and human Nude/SCID and in the DiGeorge syndromes" ;
- Call for Application in Progetti di Ricerca Scientifica Finanziabili (L.R. N.5 del 28.03.2002), with a project entitled: "Realizzazione di uno "scaffold" tridimensionale di policaprolattone per la generazione in vitro di linfociti T maturi a partire da cellule staminali".
- Fondazione Cariplo – Call for Application in Ricerca Scientifica in ambito biomedico 2009, with a project entitled: "Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood".
- Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale – Call for MIUR Application 2008, with a project entitled: "Studio delle cellule T regolatorie e delle cellule Th17 nelle immunodeficienze primitive con autoimmunità".

- Call for AIP Application 2008 with a project entitled: “Clinical and molecular characterization of pediatric patients affected with APECED: identification of functional and genetic factors influencing the phenotype expression of the disease”.
- Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale – Call for MIUR Application 2009, with a project entitled: “Studio dell'ontogenesi delle : allestimento di "scaffold" tridimensionali per la generazione in vitro di cellule T e Treg da precursori ematopoietici.”
- Fondazione Cariplo – Call for application in Ricerca Scientifica in ambito biomedico 2010, with a project entitled: “Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood”.
- “TELETHON GRANT PROPOSALS-Call for Applications 2010” with a project entitled: “Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes”.