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PhD Thesis

Immunedeficiencies: new scenarios in pathogenesis and therapeutic approaches

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INDEX

BACKGROUND AND AIM

The immune system is an interactive network, including lymphoid organs, cells, humoral factors, and cytokines, which works synergistically in host defense from pathogens, such as viruses, bacteria, fungi, and parasites, and other foreign molecules. The immune system is divided into two parts, named the innate and the adaptive immunity, which differ in speed and specificity of response (1). The innate immune response is a rapid but non-specific response, which serves as first line of defense against many common microorganisms. The innate immune system is composed of physical barriers, chemical and microbiological barriers, but it also includes cellular components, such as monocytes, macrophages, and neutrophils. However, in the case that the innate response fails to eliminate microorganisms and resultant infections, the adaptive immune system is activate through the specific cytokines **(**1, 2). The adaptive immune response is a second, more specific, line of defense against pathogens and takes several days to even weeks to develop. Compared to the innate response, adaptive immunity has immunologic "memory" of pathogenic organisms, involving antigen-specific recognition. The adaptive immunity involves specialized cells, named B and T lymphocytes. B lymphocytes are responsible for humoral immunity, through the production of antibodies, while T lymphocytes are involved in the cell-mediated immunity. Although the innate and adaptive immune systems have unique functions, their components interact and work together to protect the body from infection and disease (1, 2). A disruption of this orchestrated process leads immunedeficiencies, allergy, autoimmune disease and tumors (3).

Primary immunodeficiencies (PIDs) are genetic disorders causing severe defects in different components of immune system (4) (**Figure 1**).

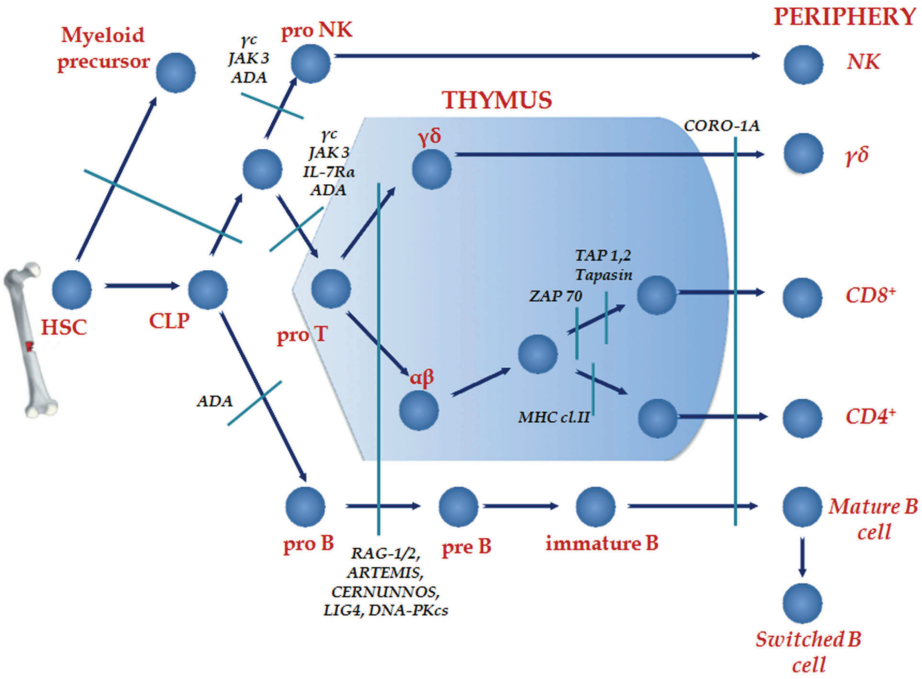

Figure 1: Genetic defects leading Immunedeficiencies.

Patients affected with PIDs are highly susceptible to severe and recurrent infections and immune reconstituting treatments are only therapeutic approach, which ensures these patients a long-term survival (5). Nowadays, more than 200 well characterized genetic immunedeficiencies have been identified thanks to the advances in molecular genetics and immunology. PIDs are classified according to the component of the immune system that is primarily involved, including T, B, natural killer (NK) cells, phagocytic cells, and complement proteins. PIDs are often associated with various autoimmune manifestations (6). Even though, for a long time autoimmunity and immune deficiency have been considered two opposite extremes of immune function,

they represent intertwined phenomena that reflect inadequate immune function (7,8). In these cases, the autoimmunity does not seem to be related to a defect of tolerance to self-antigens, but rather to a persistent stimulation as a result of the inability to eradicate foreign antigens (9). In addition, patients affected with particular forms of PID show an increased susceptibility to cancer. In particular, a high cancer susceptibility has been reported for some types of PIDs, including ataxia-telangiectasia (AT) and other DNA repair deficiencies, common variable immunodeficiency (CVID), Wiskott-Aldrich syndrome (WAS), selective IgA deficiency (IgAD), and hyper IgM syndromes (HIGM). Within PIDs, the Severe combined immunodeficiency diseases (SCIDs) represent a spectrum of illnesses with similar clinical manifestations, which can be divided into several categories on the basis of the presence or absence of T cells, B cells and Natural Killer (NK) cells. These are relatively rare diseases, collectively occurring in 1:100.000 live births. Without immune reconstitution by hematopoietic stem cell transplantation or gene therapy, these patients die of infections before 1 year of age. Fortunately, the bone marrow transplantation (BMT) HLA-identical donor (RID) is the optimal treatment for patients affected with SCIDs (10). To date, more than 20 different genetic defects involved in the pathogenesis of SCID in humans have been identified (11, 12). Typically, patients with SCID show a severe defect in T-cell differentiation and a direct or indirect impairment of B-cell development and function. On the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical phenotypes, SCIDs have been till now classified according to the presence or absence of T, B, and NK cells (**Table 1**).

SCID form	Phenotype	Transmission	Gene defect	Pathogenetic mechanism
JAK3 deficiency **γ-chain deficiency** **IL-7Rα deficiency**	$T^{-}B^{+}NK^{-}$ $T^{-}B^{+}NK^{+}$	AR	*JAK 3* *γc* *IL-7Rα*	Impaired cytokine signaling
ADA deficiency **PNP deficiency**	$T^{-}B^{-}NK^{-}$ $T^{-}B^{+}NK^{-}$	AR	*ADA* *PNP*	Accumulation of toxic metabolites
RAG 1, RAG 2 deficiency	$T^{-}B^{-}NK^{+}$	AR	*RAG1/RAG2*	Defective V(D)J recombination
ARTEMIS, KU70/80 DNA PK-cs, DNAligaseIV deficiency	$T^{-}B^{-}NK^{+}$	AR	*ARTEMIS, KU70/80 DNA PK-cs, LIG4*	Defective V(D)J recombination and DNA double-strand break repair
CERNUNNOS/XLF deficiency	T^{low} $B^{low}NK^{+}$	AR	*CERNUNNOS/XLF*	Defective V(D)J recombination and DNA double-strand break repair
Reticular Dysgenesis	$T^{-}B^{-/+}NK^{-}$	AR	*AK 2*	Increased apoptosis
ZAP 70 deficiency	$T^{-}B^{+}NK^{+}$	AR	*ZAP70*	Impaired T cell receptor signaling
MHC I deficiency **MHC II deficiency**	$T^{+}B^{+}NK^{+}$ $T^{low}B^{+}NK^{+}$	AR	*TAP1/TAP2, TAPASIN* *HLA-DR, HLA-DP, HLA-DQ, HLA-DM, HLA-DO*	Defective antigen presentation
CORO 1A deficiency	$T^{-}B^{-}NK^{+}$	AR	*CORO1A*	Abnormal actin polymerization
DOCK 8 deficiency	$T^{-}B^{+}NK^{+}$	AR	*DOCK 8*	Defective actin polymerization
Nude/SCID	$T^{-/low}B^{+}NK^{+}$	AR	*FOXN 1*	Defective intrathymic cross-talk

OS: Omenn syndrome; CID: combined immunodeficiency; HIES: hyper IgE syndrome.

Table 1: SCIDs classification according the molecular alterations and T, B, NK phenotype.

Most of the pathogenic mechanisms underlying SCIDs are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, during the last years new non-hematopoietic causative genes of novel forms of SCIDs, involving organs different from immune system, have been identified. A prototype of SCID not primarily related to hematopoietic cell abnormality but rather to an intrinsic thymic epithelial cell defect is the Nude/SCID syndrome, identified in humans for the first time in 1996. This syndrome represents the human equivalent of the *nude* murine phenotype, described by Flanagan in 1966. In humans as in mice and rats (13), the Nude/SCID phenotype results from alterations of the *FOXN1* gene (known in rats as *Whn*) (14, 15). The identification of this phenotype contributed to unravel important issues of T-cell ontogeny.

In the future, further studies on PIDs will continue to unravel novel molecular and cellular mechanisms regulating development and function of several components of immune system and the complex mechanisms involved in autoimmunity and in immune surveillance against pathogens and tumoral cells.

In this context my PhD program has been focused to the study of some Immunological disorders, in order to identify new scenarios in pathogenesis and therapeutic approaches.

This thesis reports the results obtained during my PhD course in "Human Reproduction, Development and Growth" (XXVI Cycle) from 2011 to 2014. During the past 3 years, my research has been focused in the study of the following lines of research:

- ✓ functional role of FOXN1 transcription factor in the T-cell ontogeny;

- ✓ role of common gamma chain (γc) (X-SCID causing gene) in cell cycle progression and survival of continuous and primary human malignant cell line, clarifying its involvement in leukemogenesis;
- ✓ forms of immunedeficiencies leading autoimmunity and cancer;
- ✓ rare genetic syndrome involving immune system.

CHAPTER I

"FOXN1 and T-cell development"

The thymus is a complex organ with a central role in the immune system, since it is crucially required for T-cell differentiation and repertoire selection. This organ is organized in two lobes, each one divided in two main regions: a cortical and the dark cortical area, with a high number of lymphoid cells and epithelial cells, cortical thymic epithelial cells (cTECs); a light medullary area with a low number of mature T cells, named medullary TECs (mTECs), Hassall's bodies (HB), macrophages, dendritic cells (DCs), B lymphocytes, and rarely myoid cells. Eventually, there is a transitional area, named cortico-medullary junction (CMJ), characterized by abundant blood vessels (16). These regions provide appropriate cellular interactions, cytokines and chemokines necessary to induce the fully thymocyte development. An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, that represents a heterogeneous mixture of distinct cell types, including cTECs, mTECs, fibroblasts, endothelial cells, DCs, and macrophages (17). The requirement for the 3D-supporting stroma appears to be unique to the T-cell development, as the *in vitro* differentiation program of other hematopoietic lineages, including B and NK cells, does not require a 3D structure (18). This 3D configuration of the thymus maximizes the interactions between thymic components, allowing intercellular cross-talk integral to the development of both T cells and TSCs (19).

The thymic T-cell development consists of several process that require the dynamic relocation of developing lymphocytes within multiple environments of the thymus (20). The developmental pathway is divided into three subsequent steps, as

defined by peculiar immunophenotypic patterns: the $CD4^{-}CD8^{-}$ double negative (DN) stage, the $CD4^{+}CD8^{+}$ double positive (DP) stage, and the $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ single positive (SP) stage (**Figure 2**).

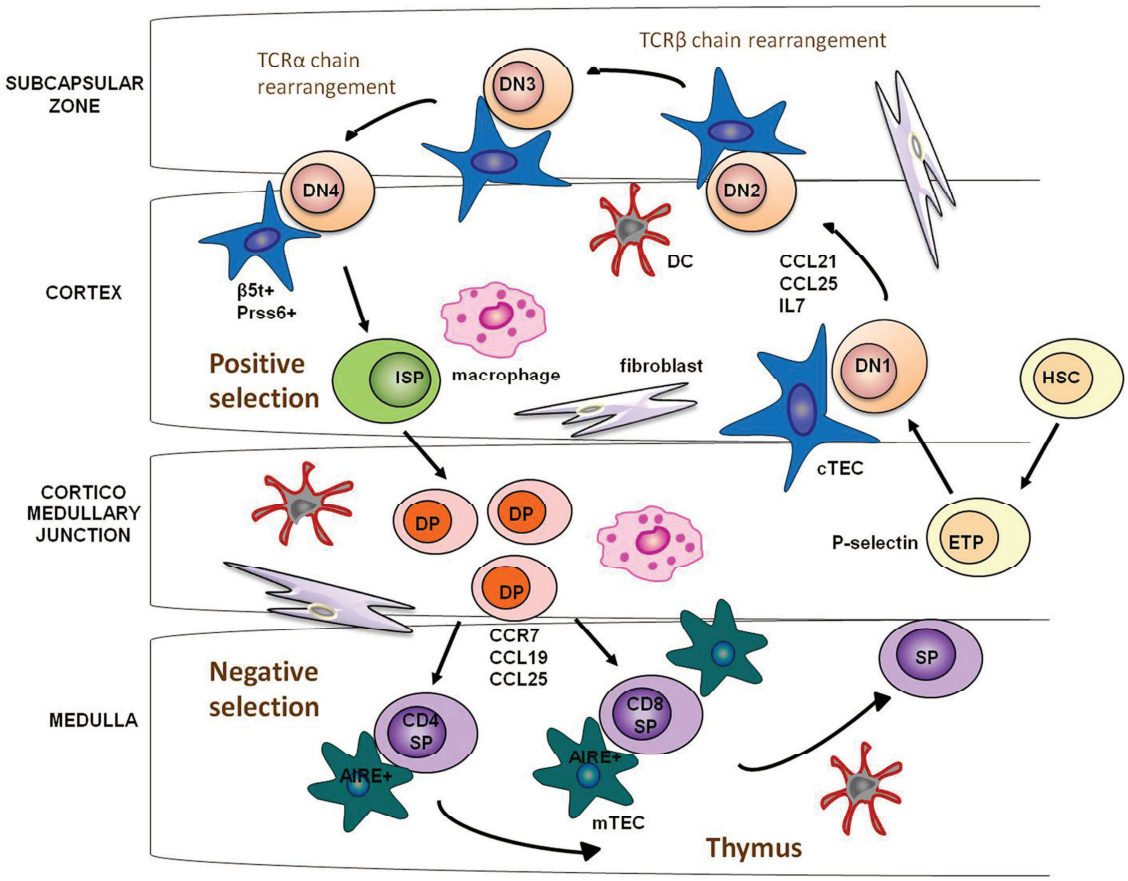

Figure 2: T-cell development and lympho-stromal crosstalk. Bone marrow HSCs enter into the thymus through CMJ, thanks to CCL19/CCL21, in embryonic thymus, and by interaction between P-selectin and its cognate ligand PSGL-1 in adult thymus. Following, IL-7 signals drive the relocation of DN thymocytes from the cortex to the subcapsular region. DP thymocytes bearing TCR and capable of binding to self-MHC ligands are positively selected. This process is regulated by Pssr6 and b5t, which are expressed in cTECs. Developing thymocytes are relocated from cortex to the medulla by chemotactic attraction between CCR7 and the ligands CCL19/CCL21, expressed on the mTECs. Into medulla, dendritic cells and Aire-expressing mTECs eliminate self-reactive thymocytes through the negative selection.

In mice, an immature single positive (ISP) $CD8^{+}CD4^{-}$ cell may be detected between the DN and DP stages. From the early T-cell lineage progenitor (ETP) stage to the double-negative 3 (DN3) stage, T-cell differentiation is independent from the TCR,

but is strictly dependent on the migration through the distinct thymic structures (21) and by the expression levels of specific transcription factors.

The DN1 cell thymocytes show the potential to differentiate into B, T,myeloid, NK, and dendritic cells (DCs) (22, 23, 24) and exhibit a high proliferative ability. The upregulation of a number of genes involved in the process, including genes needed for rearrangement and/or expression of the pre-TCR signaling complex components allows the transition to DN2 phase (25). The thymocytes at DN2 stage are fully responsive to IL-7 and SCF due to the high expression of *IL-7Rα* and *c-kit*. Moreover, the genes which favor the myeloid, NK, and dendritic fate, so-called T-cell antagonists, as PU.1, stem-cell leukemia (SCL also known as TAL1), GATA binding protein-2 (GATA-2), and CCAAT-enhancer binding protein α (C/EBPα) are silenced before that β or γδ selection takes place (Figure 2) (26). During this phase only a few transcription factors, including the zinc-finger transcription factor, the tumor suppressor factor B-cell lymphoma/leukemia 11b (BCL-11b) (27), basic helix-loop-helix (bHLH) transcription factors alternative (HEBalt) (28), and, more transiently, glioma-associated oncogene 2 (GLI-2), a transcription factor involved in the sonic hedgehog signaling (29), are expressed. The DN2 thymocytes don't completely lost, the ability to differentiate into NK cells, DCs, or macrophages (30, 31).

The following DN2 to DN3 stage transition requires the expression of different arrays of genes, as Runt-related transcription factor 1-Core binding factor β (Runx1-CBFβ) complexes, the transcription factor Myb, GATA-3, and Bcl-11b, which allow full TCRβ gene rearrangement in thymocytes, that become competent to undergo β-selection (32, 33). Several important events occur during the DN2/3 transition, as the induction of recombinase activating gene-1 (Rag-1) and Rag-2, the upregulation of pre-

Tα (pTα), and the rearrangement of TCRδ and γ. CD3ε and IL-7Rα (CD127) are also upregulated at this phase (34) along with the turn-on of the lck tyrosine kinase implicated in the pre-TCR and TCR signaling (35). At this point, T-cell precursors lose their capability to follow a non-T-cell fate choice (36). The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage (37). At the DN3 stage, pre-TCR signaling results in the downregulation of CD25, pTα, Rag-1, and Rag-2, which leads to the appearance of DN4 cells. These cells are fully committed to the αβ T-cell lineage (38). After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point, the cells become double positive (DP). Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex. The newly generated DP thymocytes are localized in the cortex and express low levels of the TCRαβ complex. This DP population consists of T cells with an unselected repertoire (39, 40). Following that, positive and negative selections take place. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells (41). When TCR interacts with low-avidity with the peptide-MHC ligands, DP thymocytes receive survival signals. This process, referred to as positive selection, allows "productive" T cells to potentially react to foreign antigens, but not to self-antigens. Lately, positively selected DP thymocytes are ready to differentiate into SP cells, that is, CD4+CD8^{-} or CD4^{-}CD8^{+} and relocate into the medulla. At this site, newly generated SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator- (AIRE-) expressing mTECs (42). The cells which are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity. SP thymocytes egress from the thymus as recent thymic

emigrants (RTEs), naïve cells expressing the CD62 ligand (CD62L), also known as lymphocyte- (L-) selectin, CD69, and the CD45RA isoform (43).

Of note, the immature thymocytes journey through the thymus has also the additional effect of promoting the differentiation of thymic stromal precursors into mature thymic epithelial cells, thus playing an important role in the formation of the thymic microenvironment (44, 45).

1.1 "FOXN1: a critic developmentally-regulated transcription factor involved in T-cell ontogeny"

Thymus anlagen arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut (20, 46). The seeding with lymphoid progenitor occurs as early as embryonic day 11.5 in mice and the eighth week of gestation in humans (47, 48). In both human and mice, the primordial thymic epithelial cells are yet incompetent to fully support T-cell development and this essential capacity is dependent on the transcriptional activation of the *FOXN1* gene in the thymic epithelium (49).

FOXN1 gene, spanning about 30 kb, is localized on chromosome 17 in human and on chromosome 11 in mice. The gene is composed of nine exons, one of which (exon 1) is non-coding. Human and mouse *FOXN1* genes show identical genomic structures, with the same number, location, phase and size of introns (49). The genes located next to *FOXN1* are also identical in both species. *FOXN1* has two alternative first exons that show tissue specificity, suggesting the existence of two different promoter sequences. *FOXN1* gene encodes for a member of the forkhead/winged helix class proteins, a large family of transcriptional factors implicated in a variety of cellular processes (50, 51). Of note, more than 100 members of this family have been identified.

FOXN1 acts through its target genes to regulate the differentiation of epithelial cells, after activation through phosphorylation, that promotes its nuclear translocation. Into the nuclei it interacts with DNA as a monomer through its forkhead box (52, 53). The signaling pathways that promotes FOXN1 expression is mainly the wingless (Wnt) proteins (54) and bone morphogenetic proteins (BMPs) (55) in both autocrine and paracrine fashions (56) During embryogenesis, *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney and urinary tract (57). In adult life, *FOXN1* is mainly expressed in the thymus and skin epithelial cells, playing a critical role in differentiation and survival of these epithalia (58, 59, 13).

Maturation of the thymic epithelial meshwork during thymic organogenesis occurs in two genetic stages (60, 61). The first stage involves *FOXN1*-independent induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch. Through studies on animal models carrying molecular alterations distinct genes, the key role of several transcription factors involved in the thymus organogenesis and TEC-sublineage specification process have thus far been identified (62). In particular, several genes, including *Tbx1* (63), *Pax1*, *Pax3*, *Pax9* (64, 65, 66), *Hoxa3* (67), *Eya1*, and *Six1* (68) have been shown to play a central role in the thymus ontogeny. The second *FOXN1*-dependent genetic step involves epithelial patterning and differentiation, as precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bipotential TEC progenitor (69, 70). It has been reported, that *FOXN1* is differentially expressed during the TE-lineage specification, since it is expressed in all TECs during the prenatal life, but not in all TECs postnatally, indicating that the gene is highly developmentally regulated. In keeping with this, a different role of *FOXN1* in

pre-natal and post-natal life has been documented. In the fetal stage, *FOXN1* mainly regulates TEC patterning. Since both cTECs and mTECs arise from the same bipotential TEC progenitors and FOXN1 regulates the process of differentiation, it is straightforward to conclude that both mTECs and cTECs are equally FOXN1-dependent during fetal thymic organogenesis (71). However, in the postnatal thymus, mTECs with the keratin type $K5^+$ and $K14^+$, which are similar to epithelial stem cell markers and exhibit activity in the skin and mammary gland epithelial progenitor cells, were more sensitive to loss of *FOXN1*; while cTECs are keratin type $K8^+$ and $K18^+$, which the same as mature epithelial markers and as terminally differentiated epithelial cells in the apical layer of stratified squamous epithelium of the skin, were not sensitive to loss of *FOXN1*.

Moreover, recent reports highlighted a central role for Foxn1 in TECs homeostasis in the adult thymus and its necessary role for the functionality and survival of adult TEC progenitors (72), expressing $K5^+$ and $K14^+$ markers. This role in adult thymus seems to be exerted in cooperation with other stem cell-related genes, such as p63. Of note, the transcription factor p63, encoding for multiple isoforms (73), drives the proliferation of TEC progenitors (74). Of note, it has been hypothesized that p63 and Foxn1 could act synergistically through the formation of a p63-Foxn1 regulatory axis aimed at regulating TECs homeostasis. However, the molecular mechanism through which the proliferation regulator p63 and differentiation regulator Foxn1 collaborate in this axis are still unclear.

In the skin, *FOXN1* regulates cell growth and differentiation and it is primarily expressed in the first suprabasal layer that contains keratinocytes in the early stages of differentiation, leaving the cell cycle and initiating terminal differentiation (75). In the

hair follicle, *FOXN1* expression is restricted to a specific compartment, the supramatrical region (75), where the cells stop to proliferate and begin terminal differentiation (76). Of note, high levels of *FOXN1* mRNA were found in co-culture of skin cells along with hematopoietic precursor cells (HPCs), suggesting a role of human skin in supporting the human T-cell development (77).

These data have been published as Reviews on *Frontiers Immunology* and *International Reviews of Immunology*, for the manuscripts see below.

frontiers in IMMUNOLOGY

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FOXN1: a master regulator gene of thymic epithelial development program

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T cell ontogeny is a sophisticated process, which takes place within the thymus through a series of well-defined discrete stages. The process requires a proper lympho-stromal interaction. In particular, cortical and medullary thymic epithelial cells (cTECs, mTECs) drive T cell differentiation, education, and selection processes, while the thymocyte-dependent signals allow thymic epithelial cells (TECs) to maturate and provide an appropriate thymic microenvironment. Alterations in genes implicated in thymus organogenesis, including *Tbx1*, *Pax1*, *Pax3*, *Pax9*, *Hoxa3*, *Eya1*, and *Six1*, affect this well-orchestrated process, leading to disruption of thymic architecture. Of note, in both human and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *Forkhead-box n1* (*FOXN1*) gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage specification in that it downstream promotes transcription of genes, which, in turn, regulate TECs differentiation. In particular, *FOXN1* mainly regulates TEC patterning in the fetal stage and TEC homeostasis in the post-natal thymus. An inborn null mutation in *FOXN1* leads to Nude/severe combined immunodeficiency (SCID) phenotype in mouse, rat, and humans. In *Foxn1*$^{-/-}$ nude animals, initial formation of the primordial organ is arrested and the primordium is not colonized by hematopoietic precursors, causing a severe primary T cell immunodeficiency. In humans, the Nude/SCID phenotype is characterized by congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder. Aim of this review is to summarize all the scientific information so far available to better characterize the pivotal role of the master regulator FOXN1 transcription factor in the TEC lineage specifications and functionality.

Keywords: Foxn1 gene, TECs, thymus gland, immunodeficiency, Nude/SCID

INTRODUCTION

The thymus is the primary lymphoid organ with the unique function to produce and to maintain the pool of mature and functional T cells. This process is strictly dependent on specialized functions of thymic stromal cells (TSCs) and requires the thymus peculiar tridimensional (3D) architecture, which allows a proper intercellular cross talk (1). For a long time, the difficulty in the isolation and characterization of the thymic cellular components has limited studies on the peculiar role of individual stromal components. Novel experimental tools, including stromal cell isolation by phenotype-based cell sorting (2), dissociation and reaggregation of stromal cell subsets (3, 4), or global gene expression analysis and the evaluation of the pattern of self-antigen expression within the individual thymic epithelial cells (TECs) subset (5), allowed to acquire important knowledge on the cellular and molecular basis of thymus organogenesis and TECs functionality.

The recent discovery of disease models associated to genetic alterations of molecules implicated in thymus specification and TECs differentiation, provided new and conclusive insights regarding the pathways, the genes, and the molecular mechanism governing these processes and stromal functionality.

THE THYMUS ARCHITECTURE: REQUIREMENT OF A 3D STRUCTURE FOR A PROPER LYMPHO-EPITHELIAL CROSSTALK

The thymus provides the microenvironment essential for the development of T cells. T cell progenitors originate in the bone marrow, enter into the thymus (6, 7) and, through a series of well defined and coordinated developmental stages, differentiate, undergo selection process, and mature into functional T cells. The steps in this process are tightly regulated through a complex network of transcriptional events, specific receptor-ligand interactions, and sensitization to trophic factors, which mediate the homing, proliferation, survival, and differentiation of developing T cells (1, 8, 9).

The thymus is organized in two lobes, which are already present in mice at 21 days of thymic organogenesis and is completely organized at 1 month of post-natal life. The lobes are divided in three areas: a cortical and the dark cortical area, with a high number of lymphoid cells and epithelial cells, cortical thymic epithelial cells (cTECs); a light medullary area with a low number of mature T cells, named medullary TECs (mTECs), Hassall's bodies (HB), macrophages, dendritic cells (DCs), B lymphocytes, and

rarely myoid cells. Eventually, there is a transitional area, named cortico-medullary junction (CMJ), characterized by abundant blood vessels (10).

The unique function of the thymus in the establishment and maintenance of the T cell pool is intimately linked to this peculiar thymus architecture and to the specialized functions of the TSCs.

LYMPHO-EPITHELIAL CROSS-TALK REQUIRED FOR THYMOCYTE AND TECs DIFFERENTIATION

An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, that represents a heterogeneous mixture of distinct cell types, including cTECs, mTECs, fibroblasts, endothelial cells, DCs, and macrophages (11). Among these stromal elements, TECs are the most abundant cell types, which form a delicate 3D cellular network spanning throughout both the thymic cortex and the medulla. The requirement for the 3D-supporting stroma appears to be unique to the T cell development, as the *in vitro* differentiation program of other hematopoietic lineages, including B and NK cells, does not require a 3D structure (12).

Thymocyte development is not a cell-autonomous process, and the transition to the next stage in development relies on the proper interaction of HSCs with thymic stroma. The 3D configuration of the thymus maximizes this interaction, allowing intercellular cross-talk integral to the development of both T cells and TSCs (13). Paralleling the T cell precursor proliferation and differentiation program, immature TECs undergo a developmental sequence, resulting in the establishment of mature cTECs and mTECs organized in this 3D network. Several studies on mutant mice with an abnormal organization of thymic epithelium substantiated the concept that a reciprocal signaling between thymocytes and TSCs is required, not only for the production of mature T cells but also for the development and organization of the thymic microenvironment in a bi-directional fashion (14, 15). Mice showing a blockage of the T cell development process, in the absence of T cell receptor (TCR)-expressing cells, have a defective organization of the thymic medulla, as well (16, 17). Of note, under this condition, thymic medullary organization can be restored by the addition of mature T cells, which follows stem cell transplantation (17, 18). In adult CD3etg26 mice, lacking intra-thymic T cell precursors, a severe alteration of the cortical thymic architecture has been documented (19), even though a restoration of the architecture and TEC development in these mice can occur. Recently, the injection of either fetal or adult T-committed precursors into adult CD3etg26 mice leads to the reconstitution of thymic microenvironment, as indicated by thymocyte differentiation, organization of functional cortical and medullary areas, and generation of $Foxp3^+$ T_{reg} and $Aire^+$ mTECs (20). These data suggest that adult TECs maintain the receptivity to cross talk with thymocytes despite a prolonged absence of T cell precursors. Moreover, the absence of both thymocytes and of the 3D framework may result in changes of the keratin genes expression, thus inducing the cTECs and mTECs to undergo a de-differentiation process and to reacquire the precursor $K5^+K8^+$ cellular phenotype. Taken together, these findings suggest that signals from early $CD4^-CD8^-$ DN T cell precursors and/or their immediate progeny provide necessary signals to promote the formation of the thymic cortex, while, later in ontogeny, the differentiation of TECs into a medullary phenotype are clearly dependent on the presence of $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) thymocytes (21–23). However, the precise molecular nature of the signals provided by developing thymocytes, which lead to the generation of the thymic stromal compartment are still incompletely defined.

Eventually, a better understanding of the developmental process through which a normal thymus structure is built, is essential for a better comprehension of the intimate mechanisms which take place within the thymus to promote the T cell development *in vivo*. This knowledge may also be useful in designing future therapeutic strategies, as alterations of the thymus structure and function may result in serious health consequences, including immunodeficiency or autoimmunity.

mTECS AND cTECS ARE SPECIALIZED CELLS PLAYING A DIFFERENT ROLE IN THE T CELL EDUCATION PROCESS

T cell ontogeny is a sophisticated process, which takes place through discrete stages during which developing thymocytes dynamically relocate in different thymic areas, following a cortico-medullary gradient.

The initial colonization of the thymus anlagen by migrant lymphoid progenitors occurs at an early stage, embryonic day 11.5 (E11.5) in mice and 8 week of gestation in humans (24, 25). Studies documented that chemokines CC ligand (CCL)21 and CCL25 play a major role in the early stage of fetal thymus colonization (26, 27). Indeed, mice deficient for these chemokines or for the cognate receptors, showed a significant reduction in the number of thymocytes compared to normal mice (28). In post-natal thymus, lymphoid progenitor cells through their cell surface adhesion molecules, such as platelet-selectin glycoprotein ligand 1, interact with P-selectin, expressed on the TECs, and thanks to this interaction they are allowed to migrate from the blood into the thymic parenchyma, in correspondence of the area around the CMJ [**Figure 1**; (29)].

Entered thymocytes started to intensely proliferate and to acquire T cell hallmarks. In this phase, T cell proliferation and differentiation are triggered by a potent combination of signals provided by cTECs. Delta-like 4 (DL4), which is an essential, non-redundant ligand for Notch1 during thymic T cell development, and IL-7 are critically involved in the activation of signaling pathways, leading to the proliferation and migration of thymocytes (30–32). In particular, these intra-thymic ligands induce the development of DN $CD25^+$ cells, which migrate toward the subcapsular region of thymic cortex (33). Several chemokine receptors have been suggested to guide the migration of immature thymocytes, such as CXCR4, CCR7, and CCR9 [**Figure 1**; (34)]. In the thymic cortex DN thymocytes begin V(D)J rearrangement of their TCRβ gene. Successfully rearranged TCRβ protein, assembled with the pre-TCRα chains, forms the pre-TCR complex. Membrane expression of pre-TCR complex, along with the Delta-Notch interaction, provides the signal necessary to induce the expression of the co-receptors CD4 and CD8, as well as V-J rearrangement of the TCRα genomic region. Subsequently, DP thymocytes with a functional TCR-αβ receptor are generated [**Figure 1**; (35)].

Thymic cortex is also the area where takes place the positive selection of DP thymocytes. Positive selection is the process by

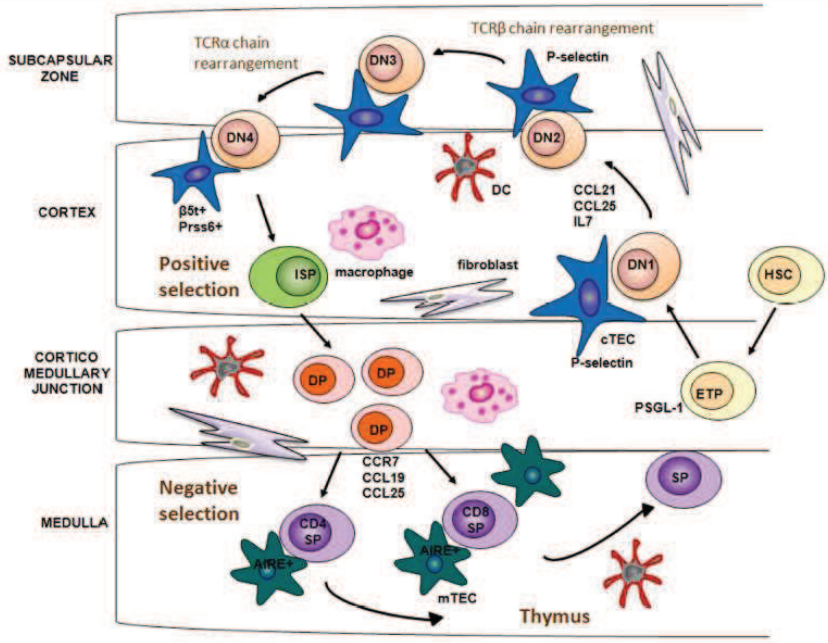

FIGURE 1 | Lympho-stromal interactions and T cell development. Bone marrow HSCs enter into the thymus through CMJ, a process mediated by CCL12 and CCL25 in the embryonic thymus and by the interaction between P-selectin and its cognate ligand PSGL-1 in adult thymus. Stimulation by IL-7 allows the relocation of DN thymocytes from the cortex to the subcapsular region. DP thymocytes bearing TCR and capable of binding to self-MHC ligands are positively selected. This process is regulated by Pssr6 and b5t, which are expressed in cTECs. Developing thymocytes are relocated from cortex to the medulla by chemotactic attraction between CCR7 and the ligands CCL19/CCL21, expressed on the mTECs. Into medulla, self-reactive thymocytes are deleted through the negative selection, a process mediated by dendritic cells and Aire-expressing mTECs.

which developing thymocytes, that recognize and bind with mild avidity peptide-major histocompatibility complex on cTECs surface, get a rescue signal through their TCR and are allowed to further maturate to the $CD4^+CD8^-$ or $CD4^-CD8^+$ SP stage. Only a small fraction (1–5%) of DP cells survive to positive selection. By contrast, the majority of DP cells, that bind with too low affinity to MHC complex, are programed to undergo death by neglect (36, 37).

Cortical thymic epithelial cells have a crucial role in the positive selection process of T cells within thymus cortex (38). Recent studies have found that cTECs exclusively express a specific form of proteasome, referred as thymoproteasome, which contains a peculiar catalytic subunit, the β5-thymus (β5t) (39). β5t subunits exhibit an unique peptidase activity, compared to other β5 subunits found in common immunoproteasome, which leads to the production of a set of self-peptides with a high affinity for class I MHC molecules (40). Moreover, β5t-deficient mice show a severe decrease in the number of $CD8^+$ SP thymocytes, but no alteration in the $CD4^+$ number or in the thymic architecture. In addition, the small fraction of $CD8^+$ T cells, positively selected by β5t-deficient cTECs, show altered immune responses toward several stimuli. Taken together these results suggest that the thymoproteasome is essential for the production of self antigens involved in the positive selection of functional $CD4^-CD8^+$ T cells (41).

As for the positive selection of $CD4^+$ T cells, two other proteins predominantly expressed in cTECs, the lysosomal protease Prss16 and Cathepsin L, have been demonstrated to be essential to generate an immunocompetent repertoire of $CD4^+CD8^-$ T cells [**Figure 1**; (42, 43)].

TCR engagement by peptide-MHC complex also triggers the expression of the chemokine receptor CCR7 in positively selected thymocytes. Thanks to the chemotactic attraction between CCR7 and its ligands, CCL19 and CCL21, expressed on the mTECs, developing thymocytes are relocated from cortex to the medulla [**Figure 1**; (44, 45)].

In order to create a repertoire of mature T cells able to recognize foreign antigens and, at the meantime, to ignore self antigens, SP thymocytes have to undergo the negative selection process in the thymic medulla. Both mTECs and DCs, play a pivotal role in this last stage of thymocyte development, which is critical to establish the central tolerance and, eventually, to prevent autoimmunity. In contrast to cTECs, mTECs are characterized by a high expression

of clustered tissue-restricted autoantigens (TSAs), the so called promiscuous gene expression (46). To date, the autoimmune regulator (AIRE) transcription factor represents the only molecule, so far identified, which contributes to the mTECs function and, in particular, to the molecular regulation of the promiscuous gene expression [**Figure 1**; (47)]. However, not all TSAs are regulated in an AIRE-dependent manner, suggesting that other molecular mechanisms, such as epigenetic mechanisms, may be involved in mTECs function regulation. TSAs associated with class II MHC molecules are presented directly by mTECs or indirectly by DCs to developing thymocytes (48). T cells which recognize with a high avidity self antigens are deleted. Remarkably, only a few number of mTECs express a given TSAs (about 50–500 per thymus), and lead to apoptosis by negative selection of a few thymocytes (37, 49, 50). A possible explanation is that the high motility of thymocytes within the thymic medulla during a period of 4–5 days, allows each of them to interact with mTECs (51). DCs play a similar role in the negative selection process. They are attracted in the thymic medulla by the chemochine XCL1 (lymphotactin), produced by mTECs in an AIRE-dependent manner. Differently from mTECs, DCs are not able to produce TSAs and the TSAs expressed mostly derive from the phagocytosis of apoptotic mTECs (52, 53). mTECs and DCs not only contribute to the establishment of central tolerance through the deletion of self-reactive T cells, but, also, through the generation of regulatory T cells (T_{regs}) (54, 55, 65, 153), which act in the periphery by suppressing autoreactive T cells, which have escaped to the process of the central tolerance.

A body of evidence documents that the expression of an autoreactive TCR leads to the entry of the thymocyte into the T_{reg} lineage. T_{regs}, that are about 5–10% of peripheral T cells $CD4^+$, constitutively express the CD25 molecule and share several immunological features, in humans and mice (56, 57). These cells specifically express the transcription factor FOXP3 (Foxp3 in mice) that plays a pivotal role in T_{regs} differentiation and function (58). The Foxp3 promoter region and the conserved non-coding sequence 2 (CNS2) (known as TSDR, the T_{reg}-specific-demethylated-region) are fully methylated in immature thymocytes (59, 155). At the beginning of T_{reg} development, an appropriate TCR/CD28 signal is needed to make available the *Foxp3* promoter through shift of the Protein Inhibitors of Activated STAT 1 (PIAS1), a signal cascade, which results in the NF-κB-mediated transcription of genes playing a role in T_{reg} differentiation (60, 61).

THYMIC FORMATION: NEW INSIGHTS IN EPITHELIAL LINEAGES SPECIFICATION

In the mouse, mTECs and cTECs originated from the third pharyngeal pouch endoderm and the thymus anlage are located next to that of the parathyroid. The expression of Forkhead-box transcription factor n1 (Foxn1) approximately at E11.5 is crucial for the subsequent epithelial differentiation, since in its absence, the colonization of the anlage by T cell progenitors from the bone marrow fails (62) and the subsequent T cell development and TECs formation is aborted, resulting in a severe immunodeficiency (63, 64, 66, 154).

The maturation process of TECs during thymic organogenesis could be divided in two genetic phases. The first stage is independent from the *Foxn1* expression and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1* and *Six* (67), *Hoxa3* (68), and *Tbx1* (69, 70). During the second genetic phase, epithelial patterning and differentiation take place and the *Foxn1* expression drives the immature epithelial cells to differentiate into functional cTECs and mTECs (71).

FOXN1-INDIPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In the first phase of the thymus organogenesis an interaction between epithelial and mesenchymal cells occurs, while at the later phase lympho-epithelial interaction predominates (72). In mice, at about E10.5 the mesenchymal cells are able to respond to the endodermic signals, which induce the development of the primordial thymic epithelium (73, 74). Subsequently, at about E12.5, the thymic rudiment is colonized by progenitors come from the fetal liver, thus resulting in a tight epithelial-thymocyte interaction within the mesenchymal derived capsule. This thymic rudiment contains the $EpCam^+Plet1^+$ epithelial population (72, 75), which includes a common thymic epithelial precursor (TEPC), from which both cTECs and mTECs will be subsequently generated (72, 76).

Through studies on animal models carrying molecular alterations of distinct genes, the key role of several transcription factors involved in the thymus organogenesis and TEC-sublineage specification process, have thus far been identified (77). In particular, several genes, including *Tbx1* (69, 70), *Pax1*, *Pax3*, *Pax9* (78–80), *Hoxa3* (68), *Eya1*, and *Six1* (67) have been shown to play a central role in the thymus ontogeny. Indeed, their molecular alteration affects this well-orchestrated process, leading to disruption of the thymic architecture. Abnormalities of the paired box (Pax) family transcription factors Pax1 or Pax9 result in a blockage of the thymus organogenesis (79, 81). Mutations in the Hox transcription factor family member, Hoxa3, expressed on both thymic epithelium and mesenchymal cells, result in athymia (68). Furthermore, the homozygous loss of *Tbx1*, related to the DiGeorge syndrome phenotype, leads to thymic a/hypoplasia in humans (69, 82), while mice heterozygous for a null allele of *Tbx1* show a mild phenotype without thymus anomalies (83). Therefore, the expression of *Tbx1* both in the pharyngeal core mesoderm and in the pharyngeal endoderm is required for a proper thymus development. However, it remains to be elucidated whether the expression of *Tbx1* in the TECs occurs and whether the gene participates in the TECs development (4).

FOXN1-DEPENDENT GENETIC STAGE OF TEC DIFFERENTIATION

In both humans and mice, the primordial TECs are yet unable to fully support T cell development and only after the transcriptional activation of the *FOXN1* gene in the thymic epithelium this essential function is acquired. *FOXN1* is a master regulator in the TEC lineage development in that it promotes down-stream the transcription of genes implicated in the thymus organogenesis and TECs full differentiation.

Forkhead-box n1 transcription factor belongs to the FOX transcription factor family implicated in a variety of biochemical and cellular processes, including development, metabolism, aging, and cancer (84, 85). During the post-natal life, *Foxn1* is selectively

expressed only in thymic and skin epithelia, where it regulates the expression of several molecular targets to maintain the balance between growth and differentiation (86, 87). The signals required for *FOXN1* expression, and its activity, are still unclear, even though the wingless (Wnt) proteins (88) and bone morphogenetic protein (BMP) signaling have been shown to regulate *FOXN1* expression (89). Even though the complete pattern of *FOXN1* expression over the time and its role are not yet completely defined, studies on mouse and human model of gene alterations enormously helped unravel important issues on its role. Mutations in *Foxn1* gene lead to alymphoid cystic thymic dysgenesis due to a defective TECs differentiation process (63, 90). In both mice and humans *FOXN1* abnormalities lead to a hairless phenotype (87, 154).

In the *Foxn1*-dependent step of thymus organogenesis, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bi-potential TEC progenitor (4, 72, 76). It has been reported, that *Foxn1* is differentially expressed during the TE-lineage specification, since it is expressed in all TECs during the pre-natal life, but not in all TECs postnatally, indicating that the gene is highly developmentally regulated. There is a body of evidence documenting different effects of *Foxn1* expression in mTEC and cTECs. Particularly, studies on K5- and K18-CreERT-mediated *Foxn1*-deleted mouse models suggested that during the post-natal life, the loss of *Foxn1* affected mTECs, characterized by the expression of K5 and K14 keratins type. Conversely, the loss of *Foxn1* did not affect cTECs, which express the keratins K8 and K18 (91, 92). Taken together, these data suggest that cTECs and mTECs are not equally *Foxn1*-dependent in the post-natal life.

Recent reports highlighted a central role for *Foxn1* in TECs homeostasis in the adult thymus and its necessary role for the functionality and survival of adult TEC progenitors (92), expressing $K5^+$ and $K14^+$ markers. This role in adult thymus seems to be exerted in cooperation with other stem cell-related genes, such as *p63*. Of note, the transcription factor p63, encoding for multiple isoforms (93), plays a pivotal for the development of stratified epithelia of several tissues, such as epidermis, breast, prostate, and thymus (94). In the thymus, the p63 protein drives the proliferation of epithelial progenitor cells (94, 95). Therefore, it has been hypothesized that *p63* and *Foxn1* could act synergistically through the formation of a *p63-Foxn1* regulatory axis aimed at regulating TECs homeostasis. However, the molecular mechanism through which the proliferation regulator *p63* and differentiation regulator *Foxn1* collaborate in this axis are still unclear.

FOXN1-MEDIATED GENE EXPRESSION FOR TEC DIFFERENTIATION

Forkhead-box n1 is directly or indirectly implicated in the transcriptional regulation of a panel of genes involved in thymus development and function.

Pax1 is a key regulator of TEC differentiation/survival balance. *Pax1* is expressed in the third pharyngeal pouch from E9.5 during the thymus ontogeny, while in the post-natal thymus only in cTEC (96). Even though the regulation of *Pax1* is still unclear, from E11.0 its expression requires *Hoxa3* (68). Of note, the loss of *Hoxa3* impairs the intrinsic ability of the neural crest cell population to differentiate and/or to lead to the differentiation of the tissues of pharyngeal arch and pouch. Indeed, in *Hoxa3* mutant mice the thymus is absent and thyroid hypoplasia has been documented (68). Moreover, the first step of thymus development is the expansion of mesenchymal neural crest in the posterior part of the third pharyngeal pouch. Prior to this event, in the *Hoxa3* mutant embryos a marked reduction in *Pax1* expression has been shown. Similarly, *Pax1* mutant mice also show thymic hypoplasia, suggesting a role for *Hoxa3* in maintaining *Pax1* expression in these cells (68). In the thymic primordium, *Pax1* expression is under the control of *Foxn1* (71). This finding indicates that *Foxn1* and *Hoxa3* are both involved in the network of molecular signals that regulates *Pax1* expression, thus demonstrating the existence of a molecular and/or functional interaction between *Hoxa3* and *Foxn1* [**Figure 2**; (71)]. In keeping with this, $Hoxa3^{+/-}Pax1^{-/-}$ compound mutant mice display a few phenotypic hallmarks of the $Foxn1^{R/R}$ mouse model, which expresses low-dose of *Foxn1*, such as hypomorphic post-natal thymus, and reduced levels of MHC class II expression on the TECs surface (80). These data suggest two alternative hypothesis: *Hoxa3* may regulate *Foxn1*, which, in turn, regulates *Pax1* expression in the thymic primordium, in a *Foxn1*-dependent manner, or *Hoxa3* and *Foxn1* induce *Pax1* expression in the third pharyngeal pouch and in early thymus primordium.

It has also been shown that *Foxn1* regulates the expression of *CCL25* and *Dll4* (**Figure 2**). These genes play a pivotal role in the thymocyte development, since *CCL25* regulates the colonization of the fetal thymus (97), while the Notch ligand *Dll4* is involved in the commitment of hematopoietic progenitors to the T cell lineage (30). In both early fetal TEC and in the post-natal thymus, *Dll4* expression is directly related to the *Foxn1* expression (71). Furthermore, these molecules are absent in the *Foxn1* null thymus, even though there is evidence indicating that their expression may occur in a *Foxn1*-independent manner in TECs (98, 99). Eventually, in a recent report it has been shown that *Foxn1* is upstream of dll4a and ccl25a expression in *medaka fish*, thus confirming the relationship with this transcription factor (100).

THE HUMAN NUDE/SCID PHENOTYPE: A MODEL OF THYMIC MICROENVIRONMENT DISRUPTION AND FAILURE OF THE T CELL DEVELOPMENT

The Nude/severe combined immunodeficiency (SCID) phenotype represents the prototype of thymic architecture disruption due to alterations of the *FOXN1*, which is the master regulator of TE-lineage specification (71).

In humans, as in mice and rats, mutations in the "nude" *Foxn1* gene induce the hairless phenotype, associated with a rudimentary thymus gland (T cell related primary immunodeficiency). The human Nude/SCID phenotype (MIM 601705; Pignata Guarino Syndrome) was first identified in 1996, after more than 30 years from the initial mouse description, in two sisters originated from a small community with a high grade of inbreeding, who showed congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T cell immunodeficiency, inherited as an autosomal recessive disorder (154). This phenotype was associated with a C792T transition in the *FOXN1* gene, which resulted in the nonsense mutation R255X in the exon 4 (formerly exon 5), with a complete absence of a functional protein similar to the previously described rat and mouse *Foxn1* mutations (101–103).

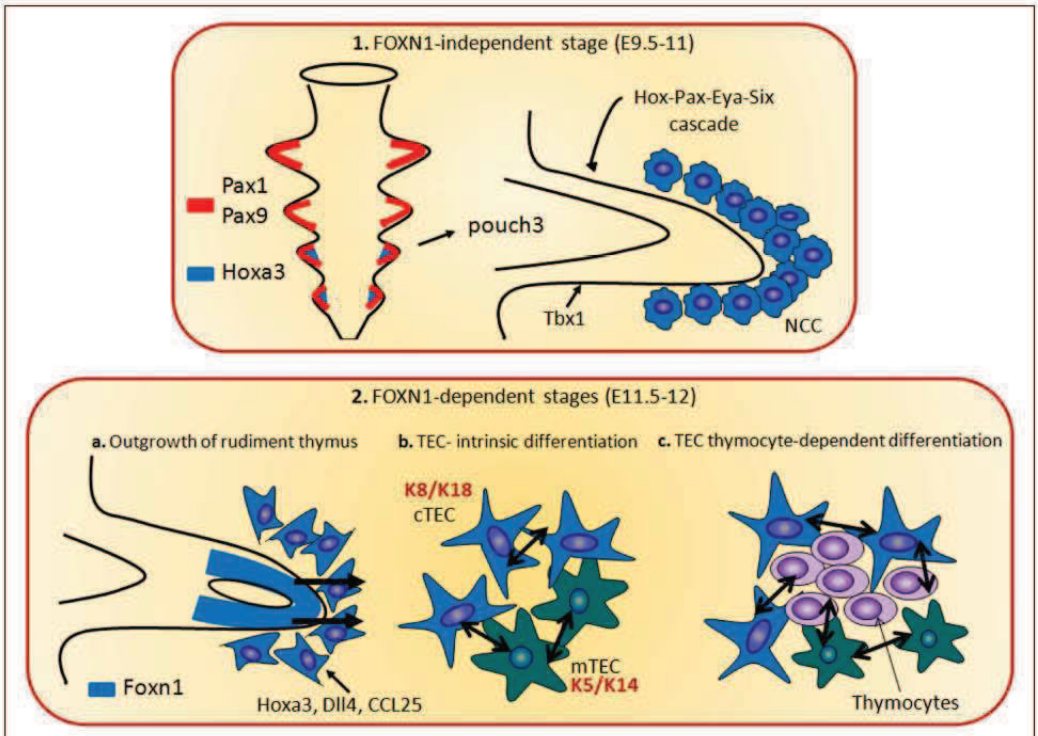

FIGURE 2 | The thymus organogenesis. The thymus organogenesis is characterized by two genetic phases. The first stage is independent from the *Foxn1* expression (1) and consists in the induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch, through the expression of genes including the *Eya1*, *Six*, *Hoxa3*, and *Tbx1*. *Hoxa3* and *Eya1* are also required in neural crest cells (NCCs). In the phase 2, *Foxn1* regulates the expression of *CCL25*, *Dll4*, and *Hoxa3*, necessary for the thymocytes and TECs differentiation. During this step, cTECs (expressing K8 and K18 keratin type) and mTECs (expressing K5 and K14 keratin type) originate from the same bi-potential TEC progenitor. The crosstalk between TECs and developing thymocytes is required to generate fully mature TECs and functional T cells.

In the absence of *Foxn1* expression, thymic development is halted at a rudimentary stage. As a consequence, in the affected patients the thymic lobe is still present but intra-thymic lymphopoiesis is completely blocked (63, 104) leading to severe primary T cell immunodeficiency (105–107) and to death in early childhood from severe infections (105, 108–112, 154). *Foxn1* is also involved in morphogenesis and maintenance of the 3D thymic micro-structure, which is necessary for a fully functional thymus (113, 114). In fact, evidence is available that in an *in vitro* 2D culture system consisting of a monolayer of mouse bone marrow stromal OP9 cells it is possible to generate mature T cells, only if these cells are transduced with the Notch ligand Delta-like 1 (OP9-DL1) (115, 116), whose pathway exerts a pivotal and necessary role in promoting the induction of T cell-lineage commitment (117–119). Of note, in all these co-culture systems, the stromal cells are enforced to overexpress Notch ligands, and their expression by TECs seems to be maintained only in a 3D thymus structure (120). In human Nude/SCID, the T cell defect is characterized by the absence of proliferative response to the common mitogens and a severe blockage of the T cell differentiation (154). Recent studies revealed the presence of some circulating T cells of non-maternal origin in patients carrying alterations of *FOXN1* gene. These cells have been shown to be predominantly double-negative αβ T cells ($CD3^+CD4^-CD8^-$, DN) and to exhibit a regulatory like T cell phenotype ($FoxP3^+$). This finding raised important issues regarding the site of differentiation of these cells. One hypothesis is the persistence of a thymic rudiment, which allows a partial T cell development (109). Alternatively, a T cell differentiation, even though partial and ineffective to result in a productive immunity, could occur at an extra-thymic site. In both pre-natal and post-natal life, the TCRBV spectratype repertoire in Nude/SCID patients is oligoclonal, thus confirming the immaturity of the process and, at the same time, that developmental events do take place at some extent (111, 112).

For many years, the human counterpart of the nude mouse phenotype has been erroneously considered the DiGeorge syndrome,

which occurs spontaneously and is mainly characterized by thymic hypo/aplasia and a mild T cell defect. However, several lines of evidence argue against the analogy between these two disorders. In fact, the 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 emerges. In addition, in this syndrome hairlessness is missing and gross abnormalities of skin annexa are not found. Children with DiGeorge syndrome may also have lymphopenia, with a mild reduction of T cells, that are however usually responsive to common mitogens.

In Nude/SCID patients, skin is tighter than usual and is characterized by basal hyperplasia and dysmaturity. Alopecia is primitive in nature, in that it can be observed at birth and persists after bone marrow transplantation, thus ruling out the acquired nature of the disorder. In keeping with this, in athymic mice, completely lacking body hair, restoration of the thymus did not lead to hair growth, indicating a direct participation of FOXN1 to hair follicle development (87). The most frequent phenotypic alteration affecting the nails is koilonychia ("spoon nail"), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; canaliform dystrophy and a transverse groove of the nail plate (Beau line) may also be observed (121). However, the most specific phenotypic alteration is leukonychia, characterized by a typical arciform pattern resembling 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. Of note, nail dystrophy has also been observed in heterozygous subjects carrying *FOXN1* alterations (121). *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 (121).

Autoptical study of a fetus homozygous for R255X mutation revealed multiple-site neural tube defects, including anencephaly and spina bifida. This finding may help explaining the high rate of mortality *in utero* observed in the population where the first patients were identified (105). Intriguingly, the other forms of SCID become clinically evident only during the postnatal life, when the protection of the newborn transferred from the mother immune system declines. This observation, suggests that other causes different from immunodeficiency, are responsible for the high rate of mortality *in utero* and led to consider the Nude/SCID mutation and anencephaly causally related. Of note, in a recent study, the mouse Foxn1 gene was found to be expressed also in epithelial cells of the developing choroids plexus, a structure filling the lateral, third and fourth ventricles of the embryonic brain (105). Moreover abnormality in the development of corpus callosum were also found in another FOXN1 mutated fetus even in the absence of anencephaly, indicating that the transcription factor may play a role as a co-factor in the brain ontogeny (105).

Altogether 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 in fetuses carrying the genetic alterations, which is not justified by the SCID *per se*.

FOXN1 MUTATION PREVENTS THE PRE-NATAL T CELL DEVELOPMENT IN HUMANS

It is now clear that FOXN1 acts as a transcription factor implicated in the differentiation of thymic and skin epithelial cells, even though many of its molecular targets still remain to be discovered. Most of the knowledge so far available has been achieved in humans in the post-natal life, while little is known about FOXN1 role during the pre-natal life.

Of note, other FOX family members, including *Foxq1* and *Foxm1b*, are important during embryogenesis, being involved in a variety of biological processes (122). Approximately 50% of *Foxq1*$^{-/-}$ murine embryos die *in utero*, thus suggesting the requirement of this gene during embryogenesis (123). Similarly, *Foxm1b* is important during liver regeneration (124).

Studies on thymus organogenesis revealed that *Foxn1* is expressed in all TECs during fetal stages. Of note, *Foxn1*$^{-/-}$ mice showed undifferentiated TECs responsible for a blockage of thymopoiesis and severe immunodeficiency (125). Recently, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study in humans the T cell development *in utero*, in the absence of a functional thymus. Vigliano et al. documented a total blockage of the CD4$^+$ T cell maturation and a severe impairment of CD8$^+$ cells, with an apparent bias toward TCRγδ$^+$ cells (112). In this case in the congenital absence of the thymus was due to R255X missense mutation in the *FOXN1* gene. In particular, it has been reported that in the absence of FOXN1 a few not functional CD8$^+$ cells, mostly bearing TCRγδ in the absence of CD3, presumably of extra-thymic origin could develop in both humans and mice (126–128). Further analysis of the fetal RNA, performed to evaluate the variable-domain β-chain (Vβ) families' usage among T lymphocytes, revealed that the generation of TCR diversity occurred at some extent in the *FOXN1*$^{-/-}$ fetus, but was abnormal. Thus, these data provided a further evidence of the crucial role for FOXN1 in the early pre-natal stages of T cell development and not in the B and NK-cell differentiation, these populations being normally present in the Nude/SCID fetus (112). A similar impairment of the T cell differentiation with a selective blockage of CD4 differentiation but not of CD8, was detected in murine models characterized by the absence of the nuclear high-mobility group (HMG) box protein TOX (107).

The identification of a limited number of CD8$^+$ cells bearing the TCRγδ suggests that this cell population may develop at extra-thymic sites in a *FOXN1*-independent manner, even though they are unable to sustain a productive immune response into the periphery. Indeed, evidence exists indicating that T cells may also differentiate at extra-thymic sites, as intestine and liver (129–133). Of note, the majority of thymus-derived T lymphocytes bears the αβ chains of TCR and a few of them express the γδ heterodimer (134), while the T cell pool developed outside the thymus is characterized by a higher proportion of TCRγδ$^+$ T cells expressing the CD8αα homodimer, instead of the CD8αβ (135, 136). Moreover, also DN T cells (CD3$^+$CD4$^-$CD8$^-$) and lymphocytes expressing CD7 and CD2 in the absence of CD3 (CD2$^+$CD3$^-$CD7$^+$) are generally considered of extra-thymic origin (135–137).

In spite of the well documented knowledge on the role of the primary lymphoid organ to foster T cell development, some still unsolved issues in human athymic conditions indicate that an in-depth information of the overall process is still to be achieved and, in particular, the involvement of different tissues in T cell ontogeny must be definitively clarified. Since FOXN1 is selectively expressed in the thymus and skin, one possibility to explain the presence of the few non-functional $CD8^+TCR\gamma\delta^+$ cells in Nude/SCID fetus is that skin epithelial cells could play a partial role in T cell ontogeny, as already shown in *in vitro* models (138, 139).

THYMUS TRANSPLANTATION: A PROMISING TREATMENT TO ATHYMIC DISORDERS

Forkhead-box n1 deficiency is a very rare immunodeficiency with unfortunately poor chance of curative treatments. Recently, thymus transplantation has emerged as a promising treatment for children affected with congenital athymia (140–143), as that observed in complete DiGeorge anomaly and in *FOXN1* deficiency. Conceptually, the thymus transplant seems to be in principle the more appropriate therapeutic strategy, taking into account that bone marrow transplantation performed in one child with *FOXN1* deficiency, failed to induce a long-term sustained immune reconstitution. In particular, in this patient no reconstitution of the naïve T cell pool was observed (144).

Thymus transplantation has been first used in children affected with complete DiGeorge anomaly, with excellent clinical and immunologic results (141). In order to achieve immune reconstitution, cultured post-natal allogeneic thymus tissue slices were transplanted into the quadriceps muscles of the athymic host (145). The migration of host bone marrow stem cells to the donor graft allow them to develop into naive T cells, which then emigrate out of the engrafted thymic tissue into the peripheral blood. Thymopoiesis is observed in biopsies of the transplanted thymus within 2 months of transplantation (140) and naive T cells are detected in the peripheral blood approximately 3–5 months after transplantation (146, 147). Taking advantage from this previous experience, a few years ago an allogeneic thymus transplantation has been used for the first time in two unrelated infants with Nude/SCID phenotype due to a deficiency of the transcription factor FOXN1 (111). The clinical phenotype of the two subjects was characterized by the absence of naïve T cells, total alopecia, nail dystrophy, and severe infections, as disseminated *Bacillus* Calmette–Guérin in subject 1 and severe respiratory infections in subject 2. Molecular analysis, performed to confirm the clinical suspect of the Nude/SCID phenotype, revealed the presence of a homozygous R255X mutation in the *FOXN1* gene in subject 1, the same of that previously described (107), and a homozygous R320W novel missense mutation in the subject 2. Moreover, subject 1 showed, like a small percentage of complete DiGeorge patients, referred as atypical complete DiGeorge, circulating oligoclonal T cells of non-maternal origins, which were predominantly double-negative T cells, and a T cell proliferative response to PHA within the normal range. Because of that, before thymus transplantation subject 1 have required immunosuppression regimen to prevent graft rejection. Differently, immunosuppression was not used for the subject 2, who had, like typical complete DiGeorge patients, very few T cells (141, 146).

Results obtained with thymus transplantation were encouraging in both FOXN1-deficient subjects, and led to a full T and B cell reconstitution and functional rescue. Indeed, both subjects developed naïve T cells, diverse TCR repertoires and an *in vitro* proliferative T cell responses against different antigens. Eventually they reached normal serum Ig levels with generation of protective antibody specific titers. Of note, HLA matching for class I and II did not seem to interfere with T cell counts after thymus transplantation, being subject 2 transplanted without any HLA matches. However, $CD8^+$ T cell number, although apparently functional, was disproportionally low compared to $CD4^+$ T cells (111). A poor CD8 recovery has also been described in complete DiGeorge patients, who underwent HLA-mismatched thymic transplantation (141, 148). Possible explanations are that the phenomenon is related to the HLA mismatch between host hematopoietic precursors and allograft thymic epithelia or to alterations in the thymic graft due to transplantation procedures.

Functionality of the thymic allograft has been assessed for the first time through signal joint (sj) and DβJβ T cell receptor rearrangement excision circle (TREC) analyses (109). The sj/βTREC represents a ratio between early and late products of TCR rearrangements, which directly correlate with thymic output and provide an indirect measurement of thymocyte division-rate (149–151). The sj/βTREC ratio quantification, conducted in subject 1 with R255X mutation, was very low during the peri-transplant period and comparable to those observed in healthy children at 2.5 years post-transplant. Of note, 4 years post-transplantation a decrease of sj/βTREC ratio associated with a reduction in sjTREC levels and in the number of naïve cells were found, suggesting the decline in thymic allograft output (109). This decline might be due to the reduced longevity of the thymus allograft or to peripheral homeostasis of the T cell pool maintenance following its replenishment. Overall, the thymus transplantation seems to be a promising curative strategy for subjects with athymia due to *FOXN1* deficiency or complete DiGeorge syndrome in the perspective of long-term clinical benefit.

CONCLUSION

The integrity of the thymic epithelial architecture allows the growth, the differentiation, and TCR repertoire selection of immature T cells, thus originating fully mature and functional T cells. Of note, the failure to generate or to maintain the proper 3D thymic architecture leads to severe immunodeficiency or autoimmunity. The unique function of the thymus in the establishment/maintenance of the T cell pool is related not only to the peculiar 3D structure, but also to the specialized functions of the thymic stroma. Indeed, lympho-stromal interactions within the multicellular thymic microenvironment play a crucial role in the regulation of the T cell development. Moreover, these interactions are based on a bilateral crosstalk between stromal cells and traveling thymocytes, which, in turn, are able to provide important signals for the TECs differentiation.

Thymus organogenesis and T cell development are sophisticated biological processes, which require the activation of a wide panel of genes. There is evidence that the master regulator of the thymus development is the *Foxn1* gene, since it is required at

multiple intermediate stages of the TE-lineage specification either in the fetal and adult thymus, through the direct or indirect regulation of genes involved in the thymus development and function. These genes include *Pax1*, *Hoxa3*, *CCL25*, *Dll4*, *p63*.

Studies on the animal and human model of the Nude/SCID phenotype have provided an enormous contribution in identifying the crucial role of *Foxn1* to drive the thymus development, even though many issues regarding the transcriptional regulation of the TECs specification and homeostasis still remain to be solved. The development *in vitro* of cellular models of TEC lineage differentiation, by using the technology of nuclear reprograming, will be certainly useful to better characterize the discrete stages of the TECs differentiation and the molecular mechanism involved in the process.

Eventually, the *in vitro* re-build of a thymic environment capable to reproduce tissue features of primary lymphoid organs (139, 152) could be a promising and valuable tool for the treatment of congenital athymia, including *FOXN1* deficiency, along with the thymus transplantation, which is emerged as a potential treatment for these disorders.

REFERENCES

1. Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol* (2001) **1**:31–40. doi:10.1038/35095500
2. Gray DH, Chidgey AP, Boyd RL. Analysis of thymic stromal cell populations using flow cytometry. *J Immunol Methods* (2002) **260**:15–28. doi:10.1016/S0022-1759(01)00493-8
3. Jenkinson EJ, Anderson G. Fetal thymic organ cultures. *Curr Opin Immunol* (1994) **6**:293–7. doi:10.1016/0952-7915(94)90104-X
4. Rodewald HR. Thymus organogenesis. *Annu Rev Immunol* (2008) **26**:355–88. doi:10.1146/annurev.immunol.26.021607.090408
5. Kyewski B, Derbinski J, Gotter J, Klein L. Promiscuous gene expression and central T-cell tolerance: more than meets the eye. *Trends Immunol* (2002) **23**:364–71. doi:10.1016/S1471-4906(02)02248-2
6. Martin CH, Aifantis I, Scimone ML, von Andrian UH, Reizis B, von Boehmer H, et al. Efficient thymic immigration of B220+ lymphoid-restricted bone marrow cells with T precursor potential. *Nat Immunol* (2003) **4**:866–73. doi:10.1038/ni965
7. Martins VC, Ruggiero E, Schlenner SM, Madan V, Schmidt M, Fink PJ, et al. Thymus-autonomous T cell development in the absence of progenitor import. *J Exp Med* (2012) **209**:1409–17. doi:10.1084/jem.20120846
8. Petrie HT, Zúñiga-Pflücker JC. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu Rev Immunol* (2007) **25**:649–79. doi:10.1146/annurev.immunol.23.021704.115715
9. Farley AM, Morris LX, Vroegindeweij E, Depreter ML, Vaidya H, Stenhouse FH, et al. Dynamics of thymus organogenesis and colonization in early human development. *Development* (2013) **140**:2015–26. doi:10.1242/dev.087320
10. Duijvestijn AM, Hoefsmit EC. Ultrastructure of the rat thymus: the micro-environment of T-lymphocyte maturation. *Cell Tissue Res* (1981) **218**:279–92. doi:10.1007/BF00210344
11. van Ewijk W. T-cell differentiation is influenced by thymic microenvironments. *Ann Rev Immunol* (1991) **9**:591–615. doi:10.1146/annurev.iy.09.040191.003111
12. Dorshkind K. Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol* (1990) **8**:111–37. doi:10.1146/annurev.immunol.8.1.111
13. Manley NR, Blackburn CC. A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? *Curr Opin Immunol* (2003) **15**:225–32. doi:10.1016/S0952-7915(03)00006-2
14. van Ewijk W, Shores EW, Singer A. Crosstalk in the mouse thymus. *Immunol Today* (1994) **15**:214–7. doi:10.1016/0167-5699(94)90246-1
15. van Ewijk W, Wang B, Hollander G, Kawamoto H, Spanopoulou E, Itoi M, et al. Thymic microenvironments, 3-D versus 2-D? *Semin Immunol* (1999) **11**:57–64. doi:10.1006/smim.1998.0158
16. Palmer DB, Viney JL, Ritter MA, Hayday AC, Owen MJ. Expression of the alpha beta T-cell receptoris necessary for the generation of the thymic medulla. *Dev Immunol* (1993) **3**:175–9. doi:10.1155/1993/56290
17. Shores EW, van Ewijk W, Singer A. Maturation of medullary thymic epithelium requires thymocytes expressing fully assembled CD3-TCR complexes. *Int Immunol* (1994) **6**:1393–402. doi:10.1093/intimm/6.9.1393
18. Surh CD, Ernst B, Sprent J. Growth of epithelial cells in the thymic medulla is under the control of mature T cells. *J Exp Med* (1992) **176**:611–6. doi:10.1084/jem.176.2.611
19. Hollander GA, Wang B, Nichogiannopoulou A, Platenburg PP, van Ewijk W, Burakoff SJ, et al. Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* (1995) **373**:350–3. doi:10.1038/373350a0
20. Roberts NA, Desanti GE, Withers DR, Scott HR, Jenkinson WE, Lane PJL, et al. Absence of thymus crosstalk in the fetus does not preclude hematopoietic induction of a functional thymus in the adult. *Eur J Immunol* (2009) **39**:2395–402. doi:10.1002/eji.200939501
21. Germeraad WT, Kawamoto H, Itoi M, Jiang Y, Amagai T, Katsura Y, et al. Development of thymic microenvironments in vitro is oxygen-dependent and requires permanent presence of T-cell progenitors. *J Histochem Cytochem* (2003) **51**:1225–35. doi:10.1177/002215540305100913
22. Gray DH, Ueno T, Chidgey AP, Malin M, Goldberg GL, Takahama Y, et al. Controlling the thymic microenvironment. *Curr Opin Immunol* (2005) **17**:137–43. doi:10.1016/j.coi.2005.02.001
23. van Ewijk W, Hollander G, Terhorst C, Wang B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* (2000) **127**:1583–91.
24. Haynes BF, Heinly CS. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. *J Exp Med* (1995) **181**:1445–58. doi:10.1084/jem.181.4.1445
25. Owen JJ, Ritter MA. Tissue interaction in the development of thymus lymphocytes. *J Exp Med* (1969) **129**:431–42. doi:10.1084/jem.129.2.431
26. Calderon L, Boehm T. Three chemokine receptors cooperatively regulate homing of hematopoietic progenitors to the embryonic mouse thymus. *Proc Natl Acad Sci U S A* (2011) **108**:7517–22. doi:10.1073/pnas.1016428108
27. Liu C, Saito F, Liu C, Lei Y, Uehara S, Love P, et al. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* (2006) **108**:2531–9. doi:10.1182/blood-2006-05-024190
28. Wurbel MA, Malissen M, Guy-Grand D, Meffre E, Nussenzweig MC, Richelme M, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes. *Blood* (2001) **98**:2626–32. doi:10.1182/blood.V98.9.2626
29. Rossi FM, Corbel SY, Merzaban JS, Carlow DA, Gossens K, Duenas J, et al. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol* (2005) **6**:626–34. doi:10.1038/ni1203
30. Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, et al. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J Exp Med* (2008) **205**:2515–23. doi:10.1084/jem.20080829
31. Thompson PK, Zúñiga-Pflücker JC. On becoming a T cell, a convergence of factors kick it up a Notch along the way. *Semin Immunol* (2011) **23**:350–9. doi:10.1016/j.smim.2011.08.007

32. Hozumi K, Mailhos C, Negishi N, Hirano K, Yahata T, Ando K, et al. Delta-like 4 is indispensable in thymic environment specific for T cell development. *J Exp Med* (2008) **205**:2507–13. doi:10.1084/jem.20080134
33. Lind EF, Prockop SE, Porritt HE, Petrie HT. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med* (2001) **194**:127–34. doi:10.1084/jem.194.2.127
34. Benz C, Heinzel K, Bleul CC. Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. *Eur J Immunol* (2004) **34**:3652–63. doi:10.1002/eji.200425248
35. Yamasaki S, Saito T. Molecular basis for pre-TCR-mediated autonomous signaling. *Trends Immunol* (2007) **28**:39–43. doi:10.1016/j.it.2006.11.006
36. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. *Nature* (1999) **402**:255–62. doi:10.1038/46218
37. Palmer E. Negative selection – clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* (2003) **3**:383–91. doi:10.1038/nri1085
38. Laufer TM, DeKoning J, Markowitz JS, Lo D, Glimcher LH. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. *Nature* (1996) **383**:81–5. doi:10.1038/383081a0
39. Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H, Takahama Y, et al. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* (2007) **316**:1349–53. doi:10.1126/science.1141915
40. Ripen AM, Nitta T, Murata S, Tanaka K, Takahama Y. Ontogeny of thymic cortical epithelial cells expressing the thymoproteasome subunit b5t. *Eur J Immunol* (2011) **41**:1278–87. doi:10.1002/eji.201041375
41. Nitta T, Murata S, Sasaki K, Fujii H, Ripen AM, Ishimaru N, et al. Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells. *Immunity* (2010) **32**:29–40. doi:10.1016/j.immuni.2009.10.009
42. Gommeaux J, Grégoire C, Nguessan P, Richelme M, Malissen M, Guerder S, et al. Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes. *Eur J Immunol* (2009) **39**:956–64. doi:10.1002/eji.200839175
43. Viret C, Lamare C, Guiraud M, Fazilleau N, Bour A, Malissen B, et al. Thymus-specific serine protease contributes to the diversification of the functional endogenous CD4 T cell receptor repertoire. *J Exp Med* (2011) **208**:3–11. doi:10.1084/jem.20100027
44. Nitta T, Nitta S, Lei Y, Lipp M, Takahama Y. CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proc Natl Acad Sci U S A* (2009) **106**:17129–33. doi:10.1073/pnas.0906956106
45. Ueno T, Saito F, Gray DH, Kuse S, Hieshima K, Nakano H, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med* (2004) **200**:493–505. doi:10.1084/jem.20040643
46. Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nature* (2001) **2**:1032–9.
47. Mathis D, Benoist C. Aire. *Annu Rev Immunol* (2009) **27**:287–312. doi:10.1146/annurev.immunol.25.022106.141532
48. Hubert FX, Kinkel SA, Davey GM, Phipson B, Mueller SN, Liston A, et al. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood* (2011) **118**:2462–72. doi:10.1182/blood-2010-06-286393
49. Derbinski J, Pinto S, Rösch S, Hexel K, Kyewski B. Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. *Proc Natl Acad Sci U S A* (2008) **105**:657–62. doi:10.1073/pnas.0707486105
50. Gillard GO, Farr AG. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J Immunol* (2006) **176**:5815–24.
51. Rooke R, Waltzinger C, Benoist C, Mathis D. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. *Immunity* (1997) **7**:123–34. doi:10.1016/S1074-7613(00)80515-4
52. Klein L. Dead man walking: how thymocytes scan the medulla. *Nat Immunol* (2009) **10**:809–11. doi:10.1038/ni0809-809
53. Koble C, Kyewski B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med* (2009) **206**:1505–13. doi:10.1084/jem.20082449
54. Lei Y, Ripen AM, Ishimaru N, Ohigashi I, Nagasawa T, Jeker LT, et al. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *J Exp Med* (2011) **208**:383–94. doi:10.1084/jem.20102327
55. Román E, Shino H, Qin FX, Liu YJ. Hematopoietic-derived APCs select regulatory T cells in thymus. *J Immunol* (2010) **185**:3819–23. doi:10.4049/jimmunol.0900665
56. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, et al. Thymus and autoimmunity: production of CD25+ CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* (1999) **162**:5317–26.
57. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. *J Exp Med* (2001) **193**:1303–10. doi:10.1084/jem.193.11.1303
58. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *J Biol Chem* (2001) **276**:37672–9. doi:10.1074/jbc.M104521200
59. Liu B, Tahk S, Yee KM, Fan G, Shuai K. The ligase PIAS1 restricts natural regulatory T cell differentiation by epigenetic repression. *Science* (2010) **330**:521–5. doi:10.1126/science.1193787
60. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, et al. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* (2007) **109**:4368–75. doi:10.1182/blood-2006-11-055756
61. Schmidt-Supprian M, Tian J, Grant EP, Pasparakis M, Maehr R, Ovaa H, et al. Differential dependence of CD4+CD25+ regulatory and natural killer like T cells on signals leading to NF-kappaB activation. *Proc Natl Acad Sci U S A* (2004) **101**:4566–71. doi:10.1073/pnas.0400885101
62. Bleul CC, Boehm T. Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol* (2000) **30**:3371–9. doi:10.1002/1521-4141(2000012)30:12<3371::AID-IMMU3371>3.0.CO;2-L
63. Nehls M, Kyewski B, Messerle M, Waldschutz R, Schuddekopf K, Smith AJ, et al. Two genetically separable steps in the differentiation of thymic epithelium. *Science* (1996) **272**:886–9. doi:10.1126/science.272.5263.886
64. Pignata C. A lesson to unraveling complex aspects of novel immunodeficiencies from the human equivalent of the nude/SCID phenotype. *J Hematother Stem Cell Res* (2002) **11**:409–14. doi:10.1089/152581602753658592
65. Pignata C, D'Agostino A, Finelli P, Fiore M, Scotese I, Cosentini E, et al. Progressive deficiencies in blood T cells associated with a 10p12-13 interstitial deletion. *Clin Immunol Immunopathol* (1996) **80**:9–15. doi:10.1006/clin.1996.0088
66. Su D, Navarre S, Oh W, Condie BG, Manley NR. A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. *Nat Immunol* (2003) **4**:1128–35. doi:10.1038/ni983
67. Zou D, Silvius D, Davenport J, Grifone R, Maire P, Xu P-X. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Dev Biol* (2006) **293**:499–512. doi:10.1016/j.ydbio.2005.12.015
68. Manley NR, Capecchi MR. The role of Hoxa-3 in mouse thymus and thyroid development. *Development* (1995) **121**:1989–2003.
69. Jerome LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat Genet* (2001) **27**:286–91. doi:10.1038/85845
70. Lindsay EA. Chromosomal microdeletions: dissecting del22q11 syndrome. *Nat Rev Genet* (2001) **2**:858–68. doi:10.1038/35098574

71. Nowell CS, Bredenkamp N, Tetelin S, Jin X, Tischner C, Vaidya H, et al. Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Genet* (2011) **7**:e1002348. doi:10.1371/journal.pgen.1002348
72. Rossi SW, Jenkinson WE, Anderson G, Jenkinson EJ. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* (2006) **441**:988–91. doi:10.1038/nature04813
73. Owen JJ, McLoughlin DE, Suniara RK, Jenkinson EJ. The role of mesenchyme in thymus development. *Curr Top Microbiol Immunol* (2000) **251**:133–7. doi:10.1007/978-3-642-57276-0_17
74. Zhang L, Sun L, Zhao Y. Thymic epithelial progenitor cells and thymus regeneration: an update. *Cell Res* (2007) **17**:50–5. doi:10.1038/sj.cr.7310114
75. Bennett AR, Farley A, Blair NF, Gordon J, Sharp L, Blackburn CC. Identification and characterization of thymic epithelial progenitor cells. *Immunity* (2002) **16**:803–14. doi:10.1016/S1074-7613(02)00321-7
76. Bleul C, Corbeaux T, Reuter A, Fisch P, Schulte Monting J, Boehm T. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* (2006) **441**:992–6. doi:10.1038/nature04850
77. Manley NR, Condie BG. Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. *Prog Mol Biol Transl Sci* (2010) **92**:103–20. doi:10.1016/S1877-1173(10)92005-X
78. Dietrich S, Gruss P. Undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. *Dev Biol* (1995) **167**:529–48. doi:10.1006/dbio.1995.1047
79. Hetzer-Egger C, Schorpp M, Haas-Assenbaum A, Balling R, Peters H, Boehm T. Thymopoiesis requires Pax9 function in thymic epithelial cells. *Eur J Immunol* (2002) **32**:1175–81. doi:10.1002/1521-4141(200204)32:4<1175::AID-IMMU1175>3.0.CO;2-U
80. Su DM, Manley NR. Hoxa3 and pax1 transcription factors regulate the ability of fetal thymic epithelial cells to promote thymocyte development. *J Immunol* (2000) **164**:5753–60.
81. Mansouri A, Goudreau G, Gruss P. Pax genes and their role in organogenesis. *Cancer Res* (1999) **59**:1707s–9.
82. Merscher S, Funke B, Epstein JA, Heyer J, Puech A, Lu MM, et al. TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome. *Cell* (2001) **104**:619–29. doi:10.1016/S0092-8674(01)00247-1
83. Liao J, Kochilas L, Nowotschin S, Arnold JS, Aggarwal VS, Epstein JA, et al. Full spectrum of malformations in velo-cardio-facial syndrome/DiGeorge syndrome mouse models by altering Tbx1 dosage. *Hum Mol Genet* (2004) **13**:1577–85. doi:10.1093/hmg/ddh176
84. Kaufmann E, Knochel W. Five years on the wings of fork head. *Mech Dev* (1996) **57**:3–20. doi:10.1016/0925-4773(96)00539-4
85. Schorpp M, Hofmann M, Dear TN, Boehm T. Characterization of mouse and human nude genes. *Immunogenetics* (1997) **46**:509–15. doi:10.1007/s002510050312
86. Brissette JL, Li J, Kamimura J, Lee D, Dotto GP. The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation. *Genes Dev* (1996) **10**:2212–21. doi:10.1101/gad.10.17.2212
87. Mecklenburg L, Tychsen B, Paus R. Learning from nudity: lessons from the nude phenotype. *Exp Dermatol* (2005) **14**:797–810. doi:10.1111/j.1600-0625.2005.00362.x
88. Balciunaite G, Keller MP, Balciunaite E, Piali L, Zuklys S, Mathieu YD, et al. Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. *Nat Immunol* (2002) **3**:1102–8. doi:10.1038/ni850
89. Bleul CC, Boehm T. BMP signaling is required for normal thymus development. *J Immunol* (2005) **175**:5213–21.
90. Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* (1994) **372**:103–7. doi:10.1038/372103a0
91. Cheng L, Guo J, Sun L, Fu J, Barnes PF, Metzger D, et al. Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy. *J Biol Chem* (2010) **285**:5836–47. doi:10.1074/jbc.M109.072124
92. Corbeaux T, Hess I, Swann JB, Kanzler B, Haas-Assenbaum A, Boehm T. Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage. *Proc Natl Acad Sci U S A* (2010) **107**:16613–8. doi:10.1073/pnas.1004623107
93. Crum CP, McKeon FD. p63 in epithelial survival, germ cell surveillance, and neoplasia. *Annu Rev Pathol* (2010) **5**:349–71. doi:10.1146/annurev-pathol-121808-102117
94. Candi E, Rufini A, Terrinoni A, Giamboi-Miraglia A, Lena AM, Mantovani R, et al. DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc Natl Acad Sci U S A* (2007) **104**:11999–2004. doi:10.1073/pnas.0703458104
95. Senoo M, Pinto F, Crum CP, McKeon F. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* (2007) **129**:523–36. doi:10.1016/j.cell.2007.02.045
96. Wallin J, Eibel H, Neubuser A, Wilting J, Koseki H, Balling R. Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* (1996) **122**:23–30.
97. Liu H, Leung BP. CD4+CD25+ regulatory T cells in health and disease. *Clin Exp Pharmacol Physiol* (2006) **33**:519–24. doi:10.1111/j.1440-1681.2006.04401.x
98. Itoi M, Tsukamoto N, Amagai T. Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus. *Int Immunol* (2007) **19**:127–32. doi:10.1093/intimm/dxl129
99. Tsukamoto N, Itoi M, Nishikawa M, Amagai T. Lack of Delta like 1 and 4 expressions in nude thymus anlages. *Cell Immunol* (2005) **234**:77–80. doi:10.1016/j.cellimm.2005.06.009
100. Bajoghli B, Aghaallaei N, Hess I, Rode I, Netuschil N, Tay BH, et al. Evolution of genetic networks underlying the emergence of thymopoiesis in vertebrates. *Cell* (2009) **138**:186–97. doi:10.1016/j.cell.2009.04.017
101. Festing MFW, May D, Connors TA, Lovell D, Sparrow S. An athymic nude mutation in the rat. *Nature* (1978) **274**:365–6. doi:10.1038/274365a0
102. Hoffman SR, Ettinger R, Zhou YJ, Gadina M, Lipsky P, Siegel R, et al. Cytokines and their role in lymphoid development, differentiation and homeostasis. *Curr Opin Allergy Clin Immunol* (2002) **2**:495–506. doi:10.1097/00130832-200212000-00004
103. Huth M, Schlake T, Boehm T. Transposon-induced splicing defect in the rat nude gene. *Immunogenetics* (1997) **45**:282–3. doi:10.1007/s002510050206
104. Blackburn CC, Augustine CL, Li R, Harvey RP, Malin MA, Boyd RL, et al. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc Natl Acad Sci U S A* (1996) **93**:5742–6. doi:10.1073/pnas.93.12.5742
105. Amorosi S, D'Armiento M, Calcagno G, Russo I, Adriani M, Christiano AM, et al. FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus. *Clin Genet* (2008) **73**:380–4. doi:10.1111/j.1399-0004.2008.00977.x
106. Cunningham-Rundles C, Ponda PP. Molecular defects in T- and B-cell primary immunodeficiency diseases. *Nat Rev Immunol* (2005) **5**:880–92. doi:10.1038/nri1713
107. Frank J, Pignata C, Panteleyev AA, Prowse DM, Baden H, Weiner L, et al. Exposing the human nude phenotype. *Nature* (1999) **398**:473–4. doi:10.1038/18997
108. Adriani M, Martinez-Mir A, Fusco F, Busiello R, Frank J, Telese S, et al. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. *Ann Hum Genet* (2004) **68**:265–8. doi:10.1046/j.1529-8817.2004.00091.x
109. Albuquerque A, Marques JG, Silva SL, Ligeiro D, Devline BH, Dutrieux J, et al. Human FOXN1-deficiency is associated with ab double-negative and FoxP3+ T-cell expansions that are distinctly modulated upon thymic transplantation. *PLoS*

ONE (2012) **7**:e37042. doi:10.1371/journal.pone.0037042
110. Coffer PJ, Burgering BMT. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* (2004) **4**:889–99. doi:10.1038/nri1488
111. Markert ML, Marques J, Neven B, Devlin B, McCarthy E, Chinn I, et al. First use of thymus transplantation therapy for Foxn1 deficiency (nude/SCID): a report of two cases. *Blood* (2011) **117**:688–96. doi:10.1182/blood-2010-06-292490
112. Vigliano I, Gorrese M, Fusco A, Vitiello L, Amorosi S, Panico L, et al. FOXN1 mutation abrogates prenatal T-cell development in humans. *J Med Genet* (2011) **48**:413–6. doi:10.1136/jmg.2011.089532
113. Mohtashami M, Zúñiga-Pflücker JC. Cutting edge: three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. *J Immunol* (2006) **176**:730–4.
114. Zúñiga-Pflücker JC. T-cell development made simple. *Nat Rev Immunol* (2004) **4**:67–72. doi:10.1038/nri1257
115. Schmitt TM, Zúñiga-Pflücker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* (2002) **17**:749–56. doi:10.1016/S1074-7613(02)00474-0
116. La Motte-Mohs RN, Herer E, Zúñiga-Pflücker JC. Induction of T cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood* (2004) **105**:1431–9. doi:10.1182/blood-2004-04-1293
117. Osborne B, Miele L. Notch and the immune system. *Immunity* (1999) **11**:653–63. doi:10.1016/S1074-7613(00)80140-5
118. MacDonald HR, Wilson A, Radtke F. Notch1 and T-cell development: insights from conditional knockout mice. *Trends Immunol* (2001) **22**:155–60. doi:10.1016/S1471-4906(00)01828-7
119. Pear WS, Radtke F. Notch signaling in lymphopoiesis. *Semin Immunol* (2003) **15**:69–79. doi:10.1016/S1044-5323(03)00003-4
120. Anderson G, Jenkinson EJ. Investigating central tolerance with reaggregate thymus organ cultures. *Methods Mol Biol* (2007) **380**:185–96. doi:10.1007/978-1-59745-395-0_11
121. Auricchio L, Adriani M, Frank J, Busiello R, Christiano A, Pignata C. Nail distrophy associated with a heterozygous mutation of the Nude/SCID human *FOXN1* (*WHN*) gene. *Arch Dermatol* (2005) **141**:647–8. doi:10.1001/archderm.141.5.647
122. Jonsson H, Peng SL. Forkhead transcription factors in immunology. *CMLS Cell Mol Life Sci* (2005) **62**:397–409. doi:10.1007/s00018-004-4365-8
123. Goering W, Adham IM, Pasche B, Manner J, Ochs M, Engel W, et al. Impairment of gastric acid secretion and increase of embryonic lethality in Foxq1-deficient mice. *Cytogenet Genome Res* (2008) **121**:88–95. doi:10.1159/000125833
124. Krupczak-Hollis K, Wang X, Kalinichenko VV, Gusarova GA, Wang IC, Dennewitz MB, et al. The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Develop Biol* (2004) **276**:74–88. doi:10.1016/j.ydbio.2004.08.022
125. Muller SM, Ege M, Pottharst A, Schulz AS, Schwarz K, Friedrich W. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. *Blood* (2001) **98**:1847–51. doi:10.1182/blood.V98.6.1847
126. Rocha B. The extrathymic T-cell differentiation in the murine gut. *Immunol Rev* (2007) **215**:166–77. doi:10.1111/j.1600-065X.2006.00467.x
127. Jensen KD, Shin S, Chien YH. Cutting edge: γδ intraepithelial lymphocytes of the small intestine are not biased toward thymic antigens. *J Immunol* (2009) **182**:7348–51. doi:10.4049/jimmunol.0900465
128. Guy-Grand D, Azogui O, Celli S, Darche S, Nussenzweig MC, Kourilsky P, et al. Extrathymic T Cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. *J Exp Med* (2003) **197**:333–41. doi:10.1084/jem.20021639
129. Blais M, Louis I, Perreault C. T-cell development: an extrathymic perspective. *Immunol Rev* (2006) **209**:103–14. doi:10.1111/j.0105-2896.2006.00341.x
130. Nonaka S, Naito T, Chen H, Yamamoto M, Moro K, Kiyono H, et al. Intestinal gd T cells develop in mice lacking thymus, all lymphonodes, Peyer's patches, and isolated lymphoid follicles. *J Immunol* (2005) **174**:1906–12.
131. Peaudecerf L, Ribeiro dos Santos P, Boudil A, Ezine S, Pardigon N, Rocha B. The role of the gut as a primary lymphoid organ: CD8 euthymic mice derive from very immature CD44+ thymocyte precursors. *Mucosal Immunol* (2011) **4**:93–101. doi:10.1038/mi.2010.47
132. Suzuki K, Oida T, Hamada H, Hitotsumatsu O, Watanabe M, Hibi T, et al. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. *Immunity* (2000) **13**:691–702. doi:10.1016/S1074-7613(00)00068-6
133. Torfadottir H, Freysdottir J, Skaftadottir I, Haraldsson A, Sigfusson G, Ogmundsdottir HM. Evidence for extrathymic T cell maturation after thymectomy in infancy. *Clin Exp Immunol* (2006) **145**:407–12. doi:10.1111/j.1365-2249.2006.03139.x
134. Xiong N, Raulet DH. Development and selection of gammadelta T cells. *Immunol Rev* (2007) **215**:15–31. doi:10.1111/j.1600-065X.2006.00478.x
135. Gunther U, Holloway JA, Gordon JG, Knight A, Chance V, Hanley NA, et al. Phenotypic characterization of CD3-7+ cells in developing human intestine and an analysis of their ability to differentiate into T cells. *J Immunol* (2005) **174**:5414–22.
136. Hayday A, Gibbons D. Brokering the peace: the origin of intestinal T cells. *Mucosal Immunol* (2008) **1**:12–4. doi:10.1038/mi.2008.8
137. Eiras P, Leon F, Camarero C, Lombardia M, Roldan E, Bootello A, et al. Intestinal intraepithelial lymphocytes contain a CD3-CD7+ subset expressing natural killer markers and a singular pattern of adhesion molecules. *Scand J Immunol* (2000) **52**:1–6. doi:10.1046/j.1365-3083.2000.00761.x
138. Aliaamad P, Kaye J. Development of all CD4 T lineages requires nuclear factor TOX. *J Exp Med* (2008) **205**:245–56. doi:10.1084/jem.20071944
139. Palamaro L, Guarino V, Scalia G, Antonini D, De Falco L, Bianchino G, et al. Human skin-derived keratinocytes and fibroblasts co-culture on 3D poly e-caprolactone scaffold support *in vitro* HSCs differentiation into T-lineage committed cells. *Int Immunol* (2013). doi:10.1093/intimm/dxt035
140. Markert ML. Treatment of infants with complete DiGeorge anomaly. *J Allergy Clin Immunol* (2008) **121**:1063. doi:10.1016/j.jaci.2007.12.1181
141. Markert ML, Devlin BH, Alexieff MJ, Li J, McCarthy EA, Gupton SE, et al. Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants. *Blood* (2007) **109**:4539–47. doi:10.1182/blood-2006-10-048652
142. Li B, Li J, Devlin BH, Markert ML. Thymic microenvironment reconstitution after postnatal human thymus transplantation. *Clin Immunol* (2011) **140**:244–59. doi:10.1016/j.clim.2011.04.004
143. Chinn IK, Markert ML. Induction of tolerance to parental parathyroid grafts using allogeneic thymus tissue in patients with DiGeorge anomaly. *J Allergy Clin* (2011) **127**:1351–2. doi:10.1016/j.jaci.2011.03.033
144. Pignata C, Gaetaniello L, Masci AM, Frank J, Christiano A, Matrecano E, et al. Human equivalent of the mouse nude/SCID phenotype: long-term evaluation of immunological reconstitution after bone marrow transplantation. *Blood* (2001) **97**:880–5. doi:10.1182/blood.V97.4.880
145. Rice HE, Skinner MA, Mahaffey SM, Oldham KT, Ing RJ, Hale LP, et al. Thymic transplantation for complete DiGeorge syndrome: medical and surgical considerations. *J Pediatr Surg* (2004) **39**:1607–15. doi:10.1016/j.jpedsurg.2004.07.020
146. Markert ML, Alexieff MJ, Li J, Sarzotti M, Ozaki DA, Devlin BH, et al. Postnatal thymus transplantation with immunosuppression as treatment for DiGeorge syndrome. *Blood* (2003) **104**:2574–81. doi:10.1182/blood-2003-08-2984

147. Markert ML, Alexieff MJ, Li J, Sarzotti M, Ozaki DA, Devlin BH, et al. Complete DiGeorge syndrome: development of rash, lymphadenopathy, and oligoclonal T cells in 5 cases. *J Allergy Clin Immunol* (2004) **113**:734–41. doi:10.1016/j.jaci.2004.01.766
148. Markert ML, Devlin BH, Chinn IK, McCarthy EA, Li YJ. Factors affecting success of thymus transplantation for complete DiGeorge anomaly. *Am J Transplant* (2008) **8**:1729–36. doi:10.1111/j.1600-6143.2008.02301.x
149. Dion ML, Poulin JF, Bordi R, Sylvestre M, Corsini R, Kettaf N, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity* (2004) **21**:757–68. doi:10.1016/j.immuni.2004.10.013
150. Dulude G, Cheynier R, Gauchat D, Abdallah A, Kettaf N, Sžkaly RP, et al. The magnitude of thymic output is genetically determined through controlled intrathymic precursor T cell proliferation. *J Immunol* (2008) **181**:7818–24.
151. Morrhaye G, Kermani H, Legros JJ, Baron F, Beguin Y, Moutschen M, et al. Impact of growth hormone (GH) deficiency and GH replacement upon thymus function in adult patients. *PLoS ONE* (2009) **4**:e5668. doi:10.1371/journal.pone.0005668
152. Clark RA, Yamanaka K, Bai M, Dowgiert R, Kupper TS. Human skin cells support thymus-indipendent T cell development. *J Clin Invest* (2005) **115**:3239–49. doi:10.1172/JCI24731
153. Klein L, Jovanovic K. Regulatory T cell lineage commitment in the thymus. *Semin Immunol* (2011) **23**:401–9. doi:10.1016/j.smim.2011.06.003
154. Pignata C, Fiore M, Guzzetta V, Castaldo A, Sebastio G, Porta F, et al. Congenital alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am J Med Genet* (1996) **65**:167–70. doi:10.1002/(SICI)1096-8628(19961016)65:2<167::AID-AJMG17>3.0.CO;2-O
155. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* (2010) **463**:808–12. doi:10.1038/nature08750

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ARTICLE

FOXN1 in Organ Development and Human Diseases

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FOXN1 gene belongs to the forkhead box gene family that comprises a diverse group of "winged-helix" transcription factors that have been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer. These transcription factors share the common property of being developmentally regulated and of directing tissue-specific transcription and cell-fate decisions. *Foxn1* is selectively expressed in thymic and skin epithelial cells, where it acts through its molecular targets to regulate the balance between growth and differentiation. In particular, *Foxn1* is required for thymic epithelial patterning and differentiation from the initial epithelial thymic anlage to a functional cortical and medullary thymic epithelial cells (TECs) meshwork necessary for the crosstalk with the lymphoid compartment. A mutation in *FoxN1* generates alymphoid cystic thymic dysgenesis due to defective TECs, causing primary T-cell immunodeficiency, named Nude/SCID syndrome, and leads to a hairless "nude" phenotype in both mice and humans. This immune defect represents the first example of a Severe Combined Immunodeficiencies (SCID) phenotype not primarily related to an abnormality intrinsic of the hematopoietic cell, but rather to a peculiar alteration of the thymic epithelia cell. This review focuses on the key role of FOXN1 in cell development and its clinical implication in humans.

Keywords FOXN1, immunodeficiency, Nude/SCID, skin, thymus

FOXN1 gene belongs to the forkhead gene family that comprises a diverse group of "winged-helix" transcription factors that have been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer [1, 2]. It was first well characterized both in mouse and humans by Schropp et al. in 1997. First designated as winged helix nude gene, because of the presence in its sequence of a forkhead domain (*Whn*), it has been then renamed forkhead box n1 (*FOXN1*). The human *FOXN1* gene is localized on chromosome 17q11–12, spanning about 30kb and being closely linked to the neurofibromatosis-1 gene [3]. Similarly to the mouse *Foxn1* gene, it contains eight coding exons and two different first exons, 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. Exon 1a and exon 1b do not contribute to the protein sequence but, interestingly, they are under the control of two promoters, which confer *FOXN1* gene tissue specificity, in that the promoter 1a is active both in thymus and skin and the promoter 1b only in the skin [3, 4].

FOXN1 gene encodes an evolutionarily high conserved forkhead/winged helix transcription factor [5], which contains 648 amino acids and exerts an 85% homology

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between rodents and humans [3]. Structural characteristics of the protein include a transcriptional activation domain, encoded by exons 8–9 and a forkhead DNA binding domain, encoded by three exons (5–7), this structure being peculiar of the forkhead/winged helix transcription factors [3]. The presence of the transactivation domain in *FOXN1* C-terminal region has been first proven by Brissette et al. [6] and then confirmed by the study of Schuddekopf et al., in which it was shown that the domain consists of approximately 50 amino acids between aa 511 and 563, rich of acidic residues [7]. Using site-directed mutagenesis, the authors replacing aspartic acid residues with alanine, found that these acidic residues are essential for the transactivation activity of the protein. Notably, not only the structural integrity of the amino acidic sequence, but also the physical proximity of both domains are essential for FOXN1 transcriptional activity [8].

Postnatally, *Foxn1* gene is mainly expressed in thymic epithelial cells (TECs), some keratinocyte populations and hair follicles, where it acts through its molecular targets to regulate the balance between growth and differentiation [6, 9, 10]. It exerts its function after activation through phosphorylation that promotes its nuclear translocation [11–13]. 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 [3, 14].

The molecular mechanisms by which FOXN1 expression and activity are regulated are not fully understood, even though evidence suggests that FOXN1 expression is strongly regulated by wingless (Wnt) proteins [15], a family of secreted glycoproteins that play a role in the cell-fate specification [16], and bone morphogenetic protein (BMP) signaling, required for a normal thymus development [17], in both autocrine and paracrine fashion (Figure 1) [15]. Foxn1 target genes have not been completely identified due to technical difficulties in isolating physiologically intact TECs at different stages of the differentiation process, even though growing evidence is now available documenting that several genes are molecular target of Foxn1 (Figure 1), including Notch ligands, *DLL-1* and *DLL-4* [18] and the chemokines, *CCL25* and *CXCL12* [19].

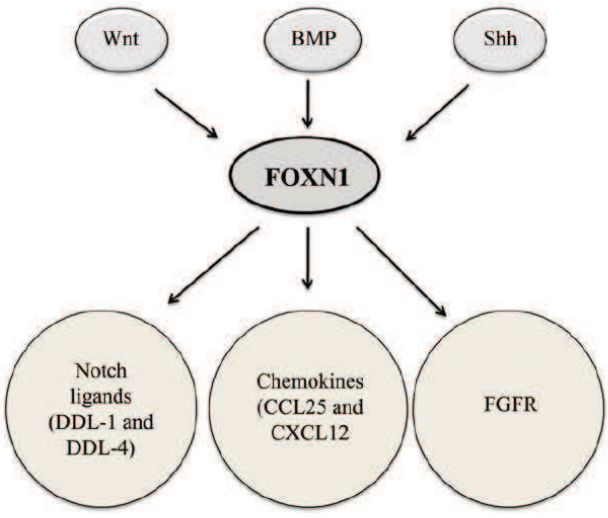

FIGURE 1. FOXN1 regulators and target genes. FOXN1 expression is regulated by wingless (Wnt) proteins, sonic hedgehog (Shh) and bone morphogenetic protein (BMP) signalings. Several genes are molecular target of Foxn1, including Notch ligands, Delta like ligand- (DLL-)1 and DLL-4, the chemokines CCL25 and CXCL12 and the fibroblast growth factor receptor (FGFR).

Moreover, it has been documented that FOXN1 up-regulates the expression level of fibroblast growth factor receptor (FGFR), which are able to regulate the differentiation of thymic epithelia and thymopoiesis, as well [16].

Even though the complete pattern of FOXN1 expression and its role are not yet completely unraveled, the knowledge of the prominent role of FOXN1 came out from studies on nude mouse and the human equivalent model determined by gene alterations. Mutation in *Foxn1* generates alymphoid cystic thymic dysgenesis due to defective TECs [5, 9] and leads to a hairless phenotype in both mouse and humans [10].

THE ROLE OF FOXN1 IN THE THYMUS

The thymus provides a specialized microenvironment for the development and selection of mature T cells bearing the T-cell receptor (TCR) complex [19, 20]. The thymic microenvironment is composed primarily of an integrated meshwork of TECs organized in three-dimensional (3D) architecture. TECs are differentiated in cortical (cTECs) and medullary epithelial cells (mTECs), required for promoting most stages of the thymocyte differentiation [21]. The intrathymic development of T cells consists of discrete phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. In the T-cell developmental pathway, hematopoietic progenitor cells (HPCs) emigrate from the bone marrow to the 3D thymus, where the developing T-cell precursors, namely, thymocytes, interact with TECs and other thymic stromal cells (TSCs). Thymocytes relocate within the organ, proliferate and acquire lineage specification. At molecular level, TCR rearrangement occurs resulting in the emigration from the thymus of a fully differentiated mature T cell [22, 23].

In particular, the epithelial cell-autonomous gene *Foxn1* is required for thymic epithelial patterning and differentiation from the initial epithelial thymic anlage to a functional cTEC and mTEC meshwork during crosstalk with the lymphoid compartment. In particular, the development and maturation of TEC subsets during the thymus organogenesis occurs through two genetic stages [9, 24], first stage being *Foxn1*-independent and under the control of genes such as *Hoxa3* [25] and *Tbx1* [26], during which induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch take place. In *Foxn1*-dependent step, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bipotential TEC progenitor [24, 27–29]. Of note, Foxn1 is expressed in all TECs during embryogenesis, but not in all TECs of the adult thymus [30], indicating that the gene is strictly developmentally regulated. However, studies on K5- and K18-CreERT-mediated *Foxn1*-deleted mouse models have revealed that *Foxn1* plays a different role in mTEC and cTECs. In particular, during the postnatal life, mTECs, expressing keratins type K5 and K14, which are similar to epithelial stem cell markers and exhibit progenitor activity in the skin and mammary gland [31, 32], are affected in the case of loss of *Foxn1*. Conversely, cTECs, whose markers are the keratins K8 and K18 expressed in terminally differentiated epithelial cells in the apical layer of stratified squamous skin epithelium, are not sensitive to the loss of *Foxn1* [33]. Thus, this evidence suggests that cTECs and mTECs are not equally *Foxn1*-dependent and cTECs are insensitive to *Foxn1* missing. Since Foxn1 is widely expressed during embryogenesis, its expression in the adult thymus reveals the presence of TEC progenitors [34], expressing $K5^+/K14^+$ markers, which support TEC homeostasis in the adult thymus.

Of note, a new component of proteasome, the β5-thymus (β5t) subunit, selectively expressed in the cTECs has been identified. β5t subunit leads to the production of peptides presented by class I MHC molecules, thus playing a pivotal role in generating the immunocompetent $CD4^-CD8^+$ T cells. In murine cTECs, the expression of β5t is

strictly dependent on *Foxn1*, which might regulate β5t transcription. These data are in keeping with the central role of this transcription factor in the stroma functionality [35].

Additionally to the well-defined role of the Foxn1 to regulate the thymic epithelial patterning and differentiation, it has recently been demonstrated that Foxn1 is also involved in the 3D thymic microstructure morphogenesis and maintenance [36], which is very important for the functionality of the thymus.

Eventually, Foxn1 must be considered as a prime downstream mediator of agents or pathways also capable to induce thymic involution or rebound. Normal aging is accompanied by thymic involution, reduced thymocyte output and reduced expression of Foxn1 [37, 38]. It has been shown that the progressive loss of Foxn1 causes accelerated premature thymic involution, with gross and microanatomic changes similar to age-related involution [38]. Furthermore, intrathymic injection of *Foxn1* cDNA to aged mice increased thymic size and thymocyte numbers [38]. Consistent with these results, down-regulation of Foxn1 occurs early in thymic involution [37], while transgenic overexpression of Foxn1 delays this process [39].

Of note, most of the pathogenic mechanisms underlying a primary T-cell disorder are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process relies on a proper crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to an alteration of the stromal component of the thymus [40].

THE ROLE OF *FOXN1* IN THE SKIN

FOXN1 gene expression in the skin, as also seen in the thymus [9], is restricted to epithelial derived cells [41]. Using *in situ* hybridization and a murine model in which a beta-galactosidase reporter gene was placed under the control of the wild-type *Foxn1* promoter, *Foxn1* gene was found to be expressed since Day 13 of gestation in the nasal region of mice. Then, *Foxn1* expression becomes detectable in developing whisker pads, nail primordial, hair follicles of eyebrows and the epidermis of mouth, nose, ears and tail. At last, *Foxn1* expression involves the entire skin, particularly interfollicular epidermis and hair follicle [41]. A detailed analysis of the *Foxn1* expression in mouse skin has revealed a specific pattern of expression, suggesting its involvement in the regulation of cell growth and differentiation.

In the interfollicular epidermis, which is composed by four cell layers (basal, spinous, granular and cornified), Foxn1 is mainly expressed within the nucleus of the cells in the first suprabasal layer. Its expression in these cells corresponds to the onset of terminal differentiation, having the cells left the cell cycle and started the full program of differentiation [41]. However, Foxn1 expression has been found, along with Ki-67 expression, also in rare cells in the basal layer. These findings allow to speculate that the double positive cells (Foxn1$^+$ Ki-67$^+$) could be markers of the very first stage of terminal differentiation, being daughter cells of transient amplifying keratinocytes, which are committed to differentiate and are just leaving the cell cycle and the basal layer [42].

In the hair follicle, Foxn1 is expressed in the supramatrical region. In particular, during hair follicle morphogenesis, its most prominent expression has been detected in the precursor cells of the inner root sheath and hair shaft, in the outer root sheath and in the periphery of the matrix [41, 43]. All these cells derive from the matrix cells after hair bulb formation and are characterized by the end of proliferation, in order to achieve their terminal differentiation [44]. After hair follicle formation, a cycle consisting of three phases takes place: anagen, hair follicle growth; catagen, hair follicle regression; telogen, resting period. Notably, Foxn1 expression is strongly dependent

on the phase of hair cycle. Indeed, the gene transcript was easily found only during anagen, while it was poorly or not at all detectable in the two further phases [43].

As far as concerned FOXN1 function in the skin, its role, its target genes and the molecular mechanism of action still remain to be fully clarified. The observation that Foxn1 is mainly expressed in the keratinocytes of the first suprabasal layer, together with the findings that nu/nu mouse keratinocytes showed a much higher susceptibility compared to wild-type keratinocytes to the growth-arresting effect of phorbol ester 12-*O*-tetradecanoyl-13-acetate (TPA), allows to speculate that FOXN1 is a key transcription factor involved in the regulation of keratinocytes growth and differentiation [6]. In addition, an increased proliferation and an impaired keratinocyte differentiation have been found in interfollicular epidermis, hair follicle and urothelium of mice, which overexpress Foxn1 in terminally differentiating cells [45]. Strong evidence suggests that Foxn1 is involved in keratinocyte differentiation through regulation of target genes, such as protein kinase C (*PKC*) and protein kinase B (*AKT*). Indeed, mouse keratinocytes, missing functional Foxn1, showed an up-regulation of PKC activity, while the overexpression of Foxn1 led to the suppression of PKC activation and inhibition of keratinocyte differentiation [46]. In addition, evidence indicates that PKC is a potent inhibitor of human hair follicle growth in vitro [47–49]. Janes et al. have investigated the function of FOXN1 in the regulation of interfollicular epidermis by means of cultures of primary human epidermal keratinocytes. They found that FOXN1 was responsible of initiation of keratinocyte differentiation but it was not sufficient to induce the final stages of terminal differentiation. Indeed, in the reconstituted human epidermis only differentiating cells of the spinal layer could be detected, while cells of the granular and cornified layers are lacking. Moreover, using microarrays analysis, approximately 30 genes have been identified, which are up-regulated following FOXN1 activation during keratinocyte differentiation. Among these genes, the serine/threonine kinase Akt is able to trigger the later stages of differentiation, leading to the development of the granular and cornified layers. Together all these findings allow to hypothesize that while FOXN1 promotes the early stage of keratinocyte differentiation, the increasing levels of AKT and its subsequent activation may bring to completion the process of terminal differentiation [50]. In keeping with this, keratinocytes from nude mice express lower level of keratin 1 (a marker of the early stages) [6], whereas overexpression of Foxn1 increases the expression keratin 1 [45, 51]. The acidic hair keratin 3 (mHa3), the murine ortholog of the human acidic hair keratin 3 and Foxn1 are coexpressed in the same anatomic structure. Reduced levels of mHa3 and other hair keratins were found in nude mice epidermis [4, 43] and transfection of FOXN1 in HeLa cells (tumoral cells derived from human cervical carcinoma) induces a high expression of mHa3, mHb3 and mHb5, hair keratins normally not expressed in this cell line [8].

Furthermore, it has been demonstrated, by using an engineered-keratin-5-driven *Foxn1* (K5-*Foxn1*) transgenic (Tg) mouse, that Foxn1 provides the necessary cues to transfer the pigment from melanocytes to keratinocytes of the hair shaft cortex, this effect being mediated by Fgf2 [52]. The *Foxn1*-null nude mouse completely lacks pigmentation in the hair cortex, while the K5-*Foxn1* Tg confers ectopic acquisition of the pigmentation in hair cortical cells.

CLINICAL IMPLICATIONS OF *FOXN1* MUTATION IN HUMANS: THE NUDE/SCID MODEL

The absence of FOXN1 transcription factor results, both in mice and humans, in congenital athymia and hairlessness. In man, the phenotype has been described in association with a C792T homozygous transition in the FOXN1 gene, which resulted in the nonsense mutation R255X in the exon 4 (formerly exon 5), with a complete absence of

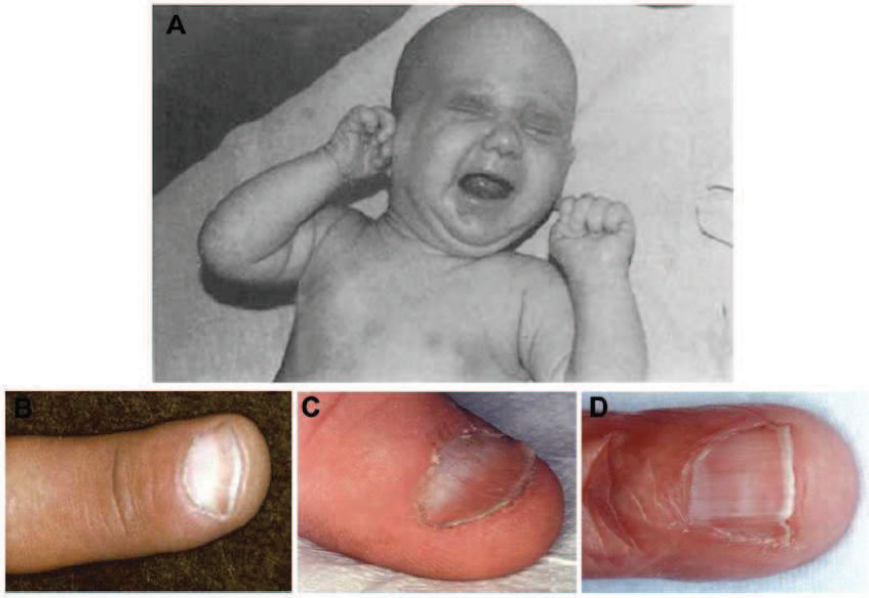

FIGURE 2. Hallmarks of the human Nude/SCID phenotype. (A) Alopecia of the scalp, eyebrows and eyelashes. Nail dystrophy patterns: (B) leukonychia, (C) koilonychias and (D) canaliform dystrophy.

a functional protein [53], or with a C987T (R320W) transition in the exon 6, resulting in a missense mutation of the DNA binding domain [54]. In particular, the genetic alteration of the transcription factor, inherited as an autosomal recessive disorder, leads to a severe T-cell immunodeficiency, congenital alopecia of scalp, eyebrows and eyelashes (Figure 2A). This phenotype, referred to as Nude/SCID (MIM 601705; Pignata Guarino Syndrome), was described for the first time in humans in 1996 in two sisters originating from a small community in the south Italy [55]. This immune defect represents the first example of a Severe Combined Immunodeficiencies (SCID) phenotype not primarily related to an abnormality intrinsic of the hematopoietic cell, but rather to a peculiar alteration of the TEC [40, 55–57].

This phenotype is widely accepted as the human equivalent of the similar murine phenotype, reported for the first time by Flanagan in 1966 [58]. Over the time, the DiGeorge syndrome (DGS), similarly characterized by thymic hypo/aplasia with subsequent impaired thymocyte development, was erroneously considered the human counterpart of nude mouse phenotype. The molecular defect responsible for the DGS is a 22q11.2 region deletion in which *Tbx1*, the gene putatively responsible for the athymia, is located. A DGS-like phenotype could also be due to other molecular defects, such as the deletion of a critical region on chromosome 10p13/14 [59, 60]. Moreover, the CHARGE syndrome, due to mutations in the *CHD7* gene encoding for a chromodomain helicase DNA-binding protein, shares considerable phenotypic overlap with DGS [61]. The spectrum of DGS defects is very heterogeneous and, in most cases, the phenotype is mild [62]. In the majority of patients with DGS, referred to as "partial," the T-cell pool is usually normal or, in very few cases, low normal, and T cells are only rarely poorly responsive to common mitogens, while patients affected with the "complete" form of the disease (accounting for 0.5%–1.5% of patients) have a severe T-cell immunodeficiency, with a naïve T-cell pool and mitogen responsiveness usually absent [63–65]. Thus, even though the Nude/SCID and a few DGS share the absence of the thymus, by a clinical and immunological point of view there are several differences, which lead to consider these disorders two completely distinct entities.

By contrast, Nude/SCID patients exhibit a quantitative and qualitative T-cell defect much more severe than that observed in DGS. The Nude/SCID immune deficiency is characterized by a severe blockage of the T-cell differentiation and absence of proliferative response to the common mitogens [55]. Naïve T cells are lacking in the peripheral blood, as a consequence of the absence of a T-cell differentiation process. In DGS, differently from Nude/SCID patients, $CD4^+CD45RA^+$ naïve cells are usually found, suggesting that a certain level of thymic functionality is still present also in the complete forms of the syndrome. The comparison of the clinical phenotype of the two syndromes reveals that in DGS hairlessness is missing and gross abnormalities of skin annexa are lacking, differently from Nude/SCID. As above described, the transcription factor FOXN1 is also expressed in the epithelial cells of the skin, where it participates to the homeostasis between growth and differentiation. This evidence argues in favor of the essential role of FOXN1 in T-cell development and leads to suggest that skin might express a major role as an alternative primary lymphoid organ. In keeping with this, it has been recently documented that in the absence of thymic cellular epithelial elements, skin-derived keratinocytes and fibroblasts seeded on the 3D scaffold, by mimicking the configuration of the thymus, are able to support the differentiation of stem cells into T-cell precursors [66].

By taking advantage of the congenital athymia, the human Nude/SCID phenotype was used as disease model to investigate the development of the T-cell compartment in the absence of a functional thymus. The results obtained from these studies revealed the presence of some circulating T cells of non-maternal origin in patients carrying alterations of FOXN1, thus raising important questions regarding the site of differentiation of these cells. These cells have been shown to be predominantly double-negative (CD4negCD8neg, DN) $\alpha\beta$ T cells and to exhibit a regulatory-like T-cell phenotype ($FoxP3^+$). One hypothesis was that a thymic rudiment could persist, allowing a partial T-cell development, but with alterations in positive/negative selection, as suggested by the expansion of DN $\alpha\beta$ and $FoxP3^+$ T cells [67]. Further evidence indicates that the *TCRBV* spectra-type repertoire in Nude/SCID patients is oligoclonal in both prenatal and postnatal life [54, 68]. Further studies aimed at characterizing the phenotype of lymphocytes in the Nude/SCID model in human prenatal life revealed that *FOXN1* alteration leads to a total blockage of $CD4^+$ T-cell maturation and severe, but not total, impairment of $CD8^+$ cell differentiation, with an apparent trend toward a $\gamma\delta$ T-cell production. This finding thus suggested a crucial role of this gene in the early prenatal stages of the T-cell ontogeny in humans [68]. Of note, different mutations in the same gene lead to different immunological phenotypes. No circulating T cells were found in a patient with the C987T (R320W) substitution identified in the exon 6 of the human FOXN1 gene, resulting in a missense mutation of the DNA binding domain [54]. These partially divergent features raise intriguing arguments about the role of FOXN1 in the T-cell development, which deserve further exploration using mouse models and comparative structural studies [4, 8, 9].

To date, there is no curative therapeutic approach for Nude/SCID syndrome. Bone marrow transplantation has been performed in one child with the Nude/SCID phenotype due to FOXN1 deficiency but without the production of the naïve T-cell pool and long-lasting immunological reconstitution [69]. This would be expected given the absence of a functional thymus and to the necessary role of the FOXN1 transcription factor for a proper T-cell development. Thus, because of the athymia in FOXN1 deficiency, an attempt to use thymus transplantation was chosen to obtain an immune reconstitution. Remarkably, naïve T cells with a diverse TCR repertoire were generated, which paralleled the normalization of T-cell proliferative responses and Ig levels. This therapeutic intervention was also useful to achieve the clearance of the ongoing disseminated infections [54]. Of note, in one patient undergone to thymus transplantation, it

has been described, for the first time, an unusual leukoderma, which the authors hypothesized as related to FOXN1 deficiency [70]. Upon transplantation of FOXN1 competent thymic epithelia, also the peripheral pool of FoxP3$^+$ T cells normalized, while the number of circulating DN$\alpha\beta$ T cells remained constantly high up to 6 years after transplantation [67]. This finding raises the question about the origin of DN$\alpha\beta$ T cells in that their reduced sjTREC levels, used as a marker of thymic functionality, suggest that they were not produced after transplantation [67], and that they probably did not require a functional thymus to develop. The evidence that other sites, alternative to the thymus, could sustain T-cell development and differentiation also derives from the identification, in a Nude/SCID fetus, of a limited number of CD8$^+$ cells, not expressing the CD3 and bearing TCR$\gamma\delta$, thus suggesting an extrathymic origin for these cells [68].

Nude/SCID phenotype is also characterized by nail abnormalities. Nail dystrophy in mice and humans is a feature of the syndrome. In man, also heterozygous gene alterations are associated with minor ectodermal anomalies, as well. Leukonychia or koilonychia ("spoon nail") are the more frequent abnormalities, the former being characterized by the typical arciform pattern resembling a half-moon and involving the proximal part of the nail plate (Figure 2B), the latter being characterized by a concave surface and significant thinning of the nail plate (Figure 2C) [71]. Also canaliform dystrophy and a transverse groove of the nail plate (Beau line) are observed, but less frequently (Figure 2D) [71]. These features are in keeping with the observation that FOXN1 is expressed in the nail matrix, thus being involved in the nail maturational process [41, 72]. Therefore, nail dystrophy could be considered an important sign to recognize heterozygous subjects [71, 73].

It should be noted that recently, multiple-site neural tube defects including anencephaly and spina bifida have been reported in a human fetus homozygous for the mutation R255X [74]. However, the analysis of a second Nude/SCID fetus did not reveal any gross abnormality in the central nervous system (CNS) anatomy but only the absence of the corpus callosum and cavum septi pellucidi (CSP), and an enlargement of the interhemispheric fissure. Of note, FOXN1 is expressed in murine epithelial cells of the developing choroids plexus in the embryonic brain [74], thus suggesting a possible role of FOXN1 in the development of the CNS in a similar fashion to other FOX family members, such as FoxP1, that helps Hox proteins regulate the genes, controlling the motor-neuron diversification [75, 76]. However, these variable abnormalities reported in the Nude/SCID phenotype suggest that FOXN1 may be implicated as a co-factor in the development of vital systems required for a proper fetus development. This would explain the high mortality rate during the first trimester of pregnancy reported among consanguineous carriers of the mutation [74], which is not justified by the SCID *per se* due to the maternal protection until the third month after birth.

CONCLUSIONS

The simultaneous occurrence of severe functional T-cell immunodeficiency and skin abnormalities associated with *FOXN1* alterations indicates that the factor exerts a critical role in the development and homeostasis of these epithelia and suggests shared functions of the gene in both thymus and skin epithelium. Evidence is available showing that a co-culture containing skin-derived cells and hematopoietic precursor cells (HPCs), reconfigured in 3D arrangement, expressing high levels of FOXN1, reproduce a thymus organoid able to generate mature and functional T cells from precursor cells even in the absence of thymic cells [77]. This central role of FOXN1 in ensuring a proper T-cell ontogeny is also indirectly sustained by the presence of functional T cells in DGS but not in Nude/SCID.

Despite the significant progress to date made, the detailed mechanism by which FOXN1 controls the T-cell differentiation process through intercellular cross-talk still remains to be clarified. Most of the target genes regulated by FOXN1 are sill undefined, also due to the technical difficulties to isolate intact TECs at different developmental stages.

Additional knowledge in this field would be very helpful in conclusively defining the role of FOXN1 in the biological process, in clarifying the intimate mechanisms of FOXN1 action and in the development of novel therapeutic strategies for congenital disorders of immune system.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Kaestner KH, Knochel W, Martinez DE. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev 2000;14:142–146.
[2] Kaufmann E, Knochel W. Five years on the wings of fork head. Mech Dev 1996;57:3–20.
[3] Schorpp M, Hofmann M, Dear TN, Boehm T. Characterization of mouse and human nude genes. Immunogenetics 1997;46:509–515.
[4] Schlake T. The nude gene and the skin. Exp Dermatol 2001;10:293–304.
[5] Nehls M, Pfeifer D, Schorpp M, et al. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. Nature 1994;372:103–107.
[6] Brissette JL, Li J, Kamimura J, et al. The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation. Genes Dev 1996;10:2212–2221.
[7] Schuddekopf K, Schorpp M, Boehm T. The Whn transcription factor encoded by the nude locus contains an evolutionarily conserved and functionally indispensable activation domain. Proc Natl Acad Sci USA 1996;93:9661–9664.
[8] Schlake T, Schorpp M, Maul-Pavicic A, et al. Forkhead/Winged-Helix transcription factor Whn regulates hair keratin gene expression: molecular analysis of the Nude skin phenotype. Dev Dyn 2000;217:368–376.
[9] Nehls M, Kyewski B, Messerle M, et al. Two genetically separable steps in the differentiation of thymic epithelium. Science 1996;272:886–889.
[10] Mecklenburg L, Tychsen B, Paus R. Learning from nudity: lessons from the nude phenotype. Exp Dermatol 2005;14:797–810.
[11] Brunet A, Bonni A, Zigmond MJ, et al. Akt prometes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 1999;96:857–868.
[12] Biggs III WH, Meisenhelder J, Hunter T, et al. Protein kinase B/Akt-mediated phosphorylation promotes nuclear, exclusion of the winged helix transcription factor FKHR1. Proc Natal Acad Sci USA 1999;96:7421–7426.
[13] Kops GJPL, de Ruiter ND, De Vries-Smits AMM, et al. Direct control of the forkhead transcription factor AFX by protein kinase B. Nature 1999;398:630–634.
[14] Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. Dev Biol 2002;250:1–23.
[15] Balciunaite G, Keller MP, Balciunaite E, et al. Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. Nat Immunol 2002;3:1102–1108.
[16] Tsai PT, Lee RA, Wu H. BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. Blood 2003;102:3947–3953.
[17] Bleul CC, Boehm T. BMP signaling is required for normal thymus development. J Immunol 2005;175:5213–5221.
[18] Tsukamoto N, Itoi M, Nishikawa M, Amagai T. Lack of Delta like 1 and 4 expressions in nude thymus anlages. Cell Immunol 2005;234:77–80.
[19] Bleul CC, Boehm T. Chemokines define distinct microenvironments in the developing thymus. Eur J Immunol 2000;30:3371–3379.
[20] Liu C, Ueno T, Kuse S, et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. Blood 2005;105:31–39.

[21] Hollander GA, Wang B, Nichogiannopoulou A, et al. Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. Nature 1995;373:350–353.
[22] van Ewijk W, Hollander G, Terhorst C, Wang B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. Development 2000;127:1583–1591.
[23] Klug DB, Carter C, Gimenez-Conti IB, Richie ER. Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. J Immunol 2002;169:2842–2845.
[24] Rodewald HR. Thymus organogenesis. Annu Rev Immunol 2008;26:355–388.
[25] Manley NR, Capecchi MR. The role of Hoxa-3 in mouse thymus and thyroid development. Development 1995;121:1989–2003.
[26] Lindsay EA, Vitelli F, Su H, et al. Tbx1 haploinsufficieny in the DiGeorge syndrome region causes aortic arch defects in mice. Nature 2001;410:97–101.
[27] Bleul CC, Corbeaux T, Reuter A, et al. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature 2006;441:992–996.
[28] Rossi SW, Jenkinson WE, Anderson G, Jenkinson EJ. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. Nature 2006;441:988–991.
[29] Anderson G, Lane PJ, Jenkinson EJ. Generating intrathymic microenvironments to establish T-cell tolerance. Nat Rev Immunol 2007;7:954–963.
[30] Itoi M, Tsukamoto N, Amagai T. Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus. Int Immunol 2007;19:127–132.
[31] Blanpain C, Lowry WE, Geoghegan A, et al. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell 2004;118:635–648.
[32] Kuraguchi M, Ohene-Baah NY, Sonkin D, et al. Genetic mechanisms in Apc-mediated mammary tumorigenesis. PLoS Genet 2009;5:e1000367.
[33] Cheng L, Guo J, Sun L, et al. Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy. J Biol Chem 2010;285:5836–5847.
[34] Corbeaux T, Hess I, Swann JB, et al. Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage. PNAS 2010;107:16613–16618.
[35] Ripen AM, Nitta T, Murata S, et al. Ontogeny of thymic cortical epithelial cells expressing the thymoproteasome subunit b5t. Eur J Immunol 2011;41:1278–1287.
[36] Guo J, Rahman M, Cheng L, et al. Morphogenesis and maintenance of the 3D thymic medulla and prevention of nude skin phenotype require FoxN1 in pre- and post-natal K14 epithelium. J Mol Med 2011;89:263–277.
[37] Ortman CL, Dittmar KA, Witte PL, Le PT. Molecular characterization of the mouse involuted thymus: abberations in expression of transcription regulators in thymocyte and epithelial compartments. Int Immunol 2002;14:813–822.
[38] Sun L, Guo J, Brown R, et al. Declining expression of a single epithelial cell-autonomous gene accelerates age-related thymic involution. Aging Cell 2010;9:347–357.
[39] Zook EC, Krishack PA, Zhang S, et al. Overexpression of FOXN1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells. Blood 2011;118:5723–5731.
[40] Pignata C. A lesson to unraveling complex aspects of novel immunodeficiencies from the human equivalent of the nude/SCID phenotype. J Hematother Stem Cell Res 2002;11:409–414.
[41] Lee D, Prowse DM, Brissette JL. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. Dev Biol 1999;208:362–374.
[42] Watt FM, Kubler MD, Hotchin NA, et al. Regulation of keratinocyte terminal differentiation by integrin-extracellular matrix interactions. J Cell Sci 1993;106:175–182.
[43] Meier N, Dear TN, Boehm T. Whn and mHa3 are components of the genetic hierarchy controlling hair follicle differentiation. Mech Dev 1999;89:215–221.
[44] Hardy MH. The secret life of the hair follicle. Trends Genet 1992;8:55–61.
[45] Prowse DM, Lee D, Weiner L, et al. Ectopic expression of the nude gene induces hyperproliferation and defects in differentiation: implications for the self-renewal of cutaneous epithelia. Dev Biol 1999;212:54–67.
[46] Li J, Baxter RM, Weiner L, et al. Foxn1 promotes keratinocyte differentiation by regulating the activity of protein kinase C. Differentiation 2007;75:694–701.
[47] Xiong Y, Harmon CS. 1beta is differentially expressed by human dermal papilla cells in response to PKC activation and is apotent inhibitor of human hair follicle growth in organ culture. J Interferon Cytokine Res 1997;17:151–157.
[48] Takahashi T, Kamimura A, Shirai A, Yokoo Y. Several selective protein kinase C inhibitors including procyanidins promote hair growth. Skin Pharmacol Appl Skin Physiol 2000;13:133–142.
[49] Harmon CS, Nevins TD, Bollag WB. Protein kinase C inhibits human hair follicle growth and hair fibre production in organ culture. Br J Dermatol 1995;133:686–693.

[50] Janes SM, Ofstad TA, Campbell DH, et al. Transient activation of FOXN1 in keratinocytes induces a transcriptional programme that promotes terminal differentiation: contrasting roles of FOXN1 and Akt. J Cell Sci 2004;117:4157–4168.
[51] Baxter RM, Brissette JL. Role of the nude gene in the epithelial terminal differentiation. J Invest Dermatol 2002;118:303–309.
[52] Weiner L, Han R, Scicchitano BM, et al. Dedicated epithelial recipient cells determine pigmentation patterns. Cell 2007;130:932–942.
[53] Frank J, Pignata C, Panteleyev AA, et al. Exposing the human nude phenotype. Nature 1999;398:473–474.
[54] Markert ML, Marques J, Neven B, et al. First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of two cases. Blood 2011;117:688–696.
[55] 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.
[56] Romano R, Palamaro L, Fusco A, et al. From murine to human Nude/SCID: the thymus, T-cell development and the missing link. Clin Dev Immunol 2012;2012:467101.
[57] Pignata C, Fusco A, Amorosi S. Human clinical phenotype associated with FOXN1 mutations. Adv Exp Med Biol 2009;665:195–206.
[58] Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. Genet Res 1966;8:295–309.
[59] Daw SC, Taylor C, Kraman M, et al. A common region of 10p deleted in DiGeorge and velocardiofacial syndromes. Nat Genet 1996;13:458–460.
[60] Monaco G, Pignata C, Rossi E, et al. DiGeorge anomaly associated with 10p deletion. Am J Med Genet 1991;39:215–216.
[61] Corsten-Janssen N, Saitta SC, Hoefsloot LH, et al. More clinical overlap between 22q11.2 deletion syndrome and charge syndrome than often anticipated. Mol Syndromol 2013;4:235–245.
[62] Digilio MC, Angioni A, De Santis M, et al. Spectrum of clinical variability in familial deletion 22q11.2: from full manifestation to extremely mild clinical anomalies. Clin Genet 2003;63:308–313.
[63] Gershwin ME. DiGeorge syndrome: congenital thymic hypoplasia. Animal model: congenitally athymic (nude) mouse. Am J Pathol 1977;89:809–812.
[64] McLean-Tooke A, Spickett GP, Gennery AR. Immunodeficiency and autoimmunity in 22q11.2 deletion syndrome. Scand J Immunol 2007;66:1–7.
[65] Ryan AK, Goodship JA, Wilson DI, et al. Spectrum of clinical features associated with interstitial chromosome 22q11 deletions: a European collaborative study. J Med Genet 1997;34:798–804.
[66] Palamaro L, Guarino V, Scalia G, et al. Human skin-derived keratinocytes and fibroblasts co-culture on 3D poly e-caprolactone scaffold support *in vitro* HSCs differentiation into T-lineage committed cells. Int Immunol 2013;25:703–714.
[67] Albuquerque A, Marques JG, Silva SL, et al. Human FOXN1-deficiency is associated with ab double-negative and FoxP3+ T-cell expansions that are distinctly modulated upon thymic transplantation. PLoS ONE 2012;7:e37042.
[68] Vigliano I, Gorrese M, Fusco A, et al. FOXN1 mutation abrogates prenatal T-cell development in humans. J Med Genet 2011;48:413–416.
[69] Pignata C, Gaetaniello L, Masci AM, et al. Human equivalent of the mouse nude/SCID phenotype: Long-term evaluation of immunological reconstitution after bone marrow transplantation. Blood 2001;97:880–885.
[70] Levy E, Neven B, Entz-Werle N, et al. Post-thymus transplant vitiligo in a child with FOXN1 deficiency. Ann Dermatol Venereol 2012;139:468–471.
[71] 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.
[72] Mecklenburg L, Paus R, Halata Z, et al. FOXN1 is critical for onycholemmal terminal differentiation in nude (Foxn1) mice. J Invest Dermatol 2004;123:1001–1011.
[73] Adriani M, Martinez-Mir A, Fusco F, et al. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. Ann Hum Genet 2004;68:265–268.
[74] Amorosi S, D'Armiento M, Calcagno G, et al. FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus. Clin Genet 2008;73:380–384.
[75] Dasen JS, De Camilli A, Wang B, et al. Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell 2008;134:304–316.
[76] Rousso DL, Gaber ZB, Wellik D, et al. Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. Neuron 2008;59:226–240.
[77] Clark RA, Yamanaka K, Bai M, et al. Human skin cells support thymus-independent T cell development. J Clin Invest 2005;115:3239–3249.

1.2. The human NUDE/SCID phenotype: a model to study T-cell development

Recessive mutations in the *FOXN1* gene lead *nude* phenotype, described for the first time in mice. This murine model was described by Flanagan in 1966, when spontaneously appeared in the Virus Laboratory of Ruchill Hospital in Glasgow (UK) (13, 51). Mice homozygous for the mutation "nude" are hairless, have retarded growth, decreased fertility, and die by 5 months of life for infections. Thymus abnormality, or even absence, is the hallmark of the *nude* phenotype. In fact, these animals develop a profound T-cell deficiency and a severely impaired immune response of either cell-mediated and, indirectly, humoral immunity (78). Today, *nude* mice are a widely use model in immunology, dermatology, cosmetic, oncological and transplantation research fields because of their defect in allo- or xeno-transplantation rejection due to the absence of a functional immune system (79). In man, the prototype of an athymic disorder has long been considered the DiGeorge's Syndrome (DGS), even though main features of athymic murine model and human disease, including immunological signs, are not completely overlapping. The human counterpart of murine *nude* phenotype was described for the first time in 1996 by our research group, as a novel form of SCID caused by an intrinsic defect of the thymus (MIM 601705) in 2 patients born to consanguineous parents originating from a small community in southern Italy. The hallmarks of this human novel clinical phenotype are ectodermal abnormalities, as alopecia and nail dystrophy (80), and a profound T-cell defect (51). Of note, the nail dystrophy can be observed also in subjects carrying the genetic alteration in heterozygosity. The most frequent nail alteration is the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; a canaliform dystrophy associated to a transverse

groove of the nail plate (Beau line) may also be found (80). The first described human *FOXN1* mutation was a C792T transition in exon 5, resulting in the nonsense mutation R255X (13). This mutation lies upstream of the DNA binding and trans-activation domain of this transcription factor, so that translated protein, if any, would be completely non-functional, similar to the previously described rat and mouse *Foxn1* mutations (81).

Recently, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study in humans the T-cell development *in utero*, in the absence of a functional thymus. *Vigliano et al.*, documented a total blockage of the CD4^{+} T-cell maturation and a severe impairment of CD8^{+} cells, with an apparent bias towards TCRγδ^{+} cells. In this case, the congenital absence of the thymus was due to R255X missense mutation in the FOXN1 gene (82). In particular, it has been reported that, in the absence of FOXN1, a few not functional CD8^{+} cells could develop, mostly bearing TCRγδ in the absence of CD3. Further analysis of the fetal RNA, performed to evaluate the variable-domain β-chain (Vβ) families' usage among T lymphocytes, revealed that the generation of TCR diversity occurred at some extent in the *FOXN1*$^{-/-}$ fetus, but was abnormal. Thus, these data provided a further evidence of the crucial role for FOXN1 in the early prenatal stages of T-cell development and not in the B- and NK-cell differentiation, these populations being normally present in the Nude/SCID fetus (82). A similar impairment of the T-cell differentiation with a selective blockage of CD4 differentiation but not of CD8, was detected in murine models characterized by the absence of the nuclear high-mobility group (HMG) box protein TOX (15).

The identification of a limited number of CD8^{+} cells bearing the TCRγδ suggests that this cell population may develop at extrathymic sites in a FOXN1-independent

manner, even though they are unable to sustain a productive immune response into the periphery. Indeed, evidence exists indicating that T cells may also differentiate at extrathymic sites, as intestine and liver (83, 84, 85). Of note, the majority of thymus-derived T lymphocytes bears the αβ chains of TCR and a few of them express the γδ heterodimer (86), while the T-cell pool developed outside the thymus is characterized by a higher proportion of TCRγδ^{+} T cells expressing the CD8αα homodimer, instead of the CD8αβ (87). Moreover, also DN T cells ($CD3^{+}CD4^{-}CD8^{-}$) and lymphocytes expressing CD7 and CD2 in the absence of CD3 ($CD2^{+}CD3^{-}CD7^{+}$) are generally considered of extrathymic origin (87, 88), suggesting the existence of extrathymic sites of thymus-independent and *FOXN1*-independent T lymphopoiesis.

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Review Article

From Murine to Human Nude/SCID: The Thymus, T-Cell Development and the Missing Link

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Primary immunodeficiencies (PIDs) are disorders of the immune system, which lead to increased susceptibility to infections. T-cell defects, which may affect T-cell development/function, are approximately 11% of reported PIDs. The pathogenic mechanisms are related to molecular alterations not only of genes selectively expressed in hematopoietic cells but also of the stromal component of the thymus that represents the primary lymphoid organ for T-cell differentiation. With this regard, the prototype of athymic disorders due to abnormal stroma is the Nude/SCID syndrome, first described in mice in 1966. In man, the DiGeorge Syndrome (DGS) has long been considered the human prototype of a severe T-cell differentiation defect. More recently, the human equivalent of the murine Nude/SCID has been described, contributing to unravel important issues of the T-cell ontogeny in humans. Both mice and human diseases are due to alterations of the FOXN1, a developmentally regulated transcription factor selectively expressed in skin and thymic epithelia.

1. Introduction

Primary immunodeficiencies (PIDs) are severe disorders of the immune system in which patients cannot produce a proper protective immune response, leading to an increased susceptibility to infections. Nowadays, more than 200 well-characterized genetic immune deficiencies have been identified thanks to the advances in molecular genetics and immunology. PIDs are classified according to the component of the immune system that is primarily involved including T, B, natural killer (NK) lymphocytes, phagocytic cells, and complement proteins [1].

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs [2]. These diseases may be considered true experiments of the nature in that the recognition of the molecular mechanisms underlying their pathogenesis led to clarify the phases of the T-cell differentiation process and the physiological mechanisms of the T-cell responses. Studies in this field led to unravel the checkpoints, which play a pivotal role in these processes, which mostly rely on a proper intercellular interaction between thymocytes and the thymic microenvironment.

2. T-Cell Development and Thymus

The thymus is the primary lymphoid organ that supports T-cell differentiation and repertoire selection [3, 4]. The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. As shown in Figure 1, these steps are (1) the entry of lymphoid progenitor cells into the thymus, (2) the generation of $CD4^+$ $CD8^+$ double positive (DP) thymocytes in the cortex, (3) the positive selection of DP thymocytes in the cortex, and (4) the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete the thymocyte maturation and, eventually, the export of mature T cells from the thymus [5].

Thymus anlagen arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut [6, 7]. The interaction of the epithelial component with the lymphoid progenitor takes place as early as embryonic day 11.5 in mice and at the eighth week of gestation in humans [8, 9].

At an early stage, these precursors have both lymphoid and myeloid potential [10, 11] and are characterized by

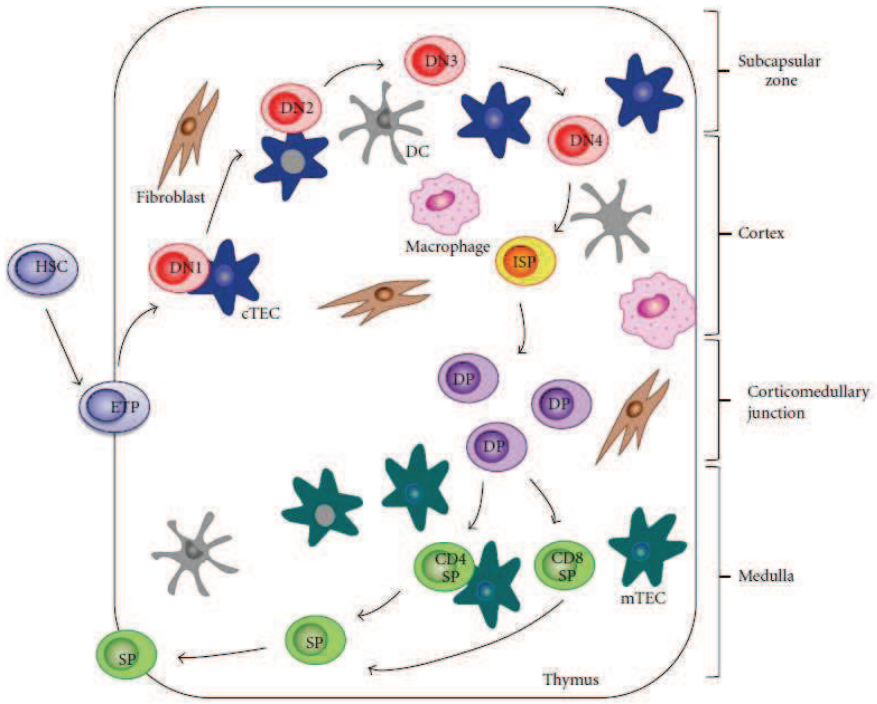

FIGURE 1: Steps of T-cell development. The lymphoid progenitor cell goes into the thymus through the cortico-medullary junction. DN thymocytes ($CD4^-CD8^-$) migrate across the subcapsular region and then the outer cortex. Interaction between DN cells and cTECs generates DP thymocytes ($CD3^+CD4^+CD8^+$). Positively selected thymocytes interact with mTECs to complete the maturation process. In the medulla, self-reactive thymocytes are deleted, SP ($CD3^+CD4^+$ or $CD3^+CD8^+$) thymocytes are generated, and, eventually, the export of mature T cells from the thymus takes place.

the expression of the CC-chemokine receptor 9 (CCR9), that, along with the CCR7, plays a central role in this precocious stage of thymus colonization. At this stage of differentiation, lymphoid cells also express the stem- and progenitor-cell markers KIT (also known as CD117), the stem-cell antigen-1 (SCA-1), and the growth-factor-receptor tyrosine kinase type 3 (FLT3) [12–14].

Following the entry into the thymus through the corticomedullary junction, lymphoid progenitor cells begin their commitment toward the T-cell lineage. The developmental pathway is traditionally divided into three subsequent steps, as defined by peculiar immunophenotypic patterns: the $CD4^-CD8^-$ double negative (DN) stage, the $CD4^+CD8^+$ double positive (DP) stage, and the $CD4^-CD8^+$ or $CD4^+CD8^-$ single positive (SP) stage. In mice, an immature single positive (ISP) $CD8^+CD4^-$ cell may be detected between the DN and DP stages. This population can be easily distinguished from the mature SP cell by the high levels of expression of T-cell receptor (TCR) β and CD3 and the low level of CD24 (heat stable antigen, HSA). DN cells in mice can be further subdivided based on the expression of CD44 and CD25 in the following populations: $CD44^+CD25^-$(DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$(DN3), and $CD44^-CD25^-$(DN4) [15].

From the early T-cell lineage progenitor (ETP) stage to the double-negative 3 (DN3) stage, T-cell differentiation is independent from the TCR and is dependent on the migration through the distinct thymic structures [16]. These phases are regulated by the expression levels of specific transcription factors and by a fine tuned interplay between them (Figure 1).

At the beginning, ETPs and DN2 cells exhibit a high proliferative capability. Differently, at the DN3 stage, when a fully rearranged TCR occurs, the proliferation stops. In the initial thymocyte development till the DN3 stage, Notch-mediated signals play a pivotal role [17, 18] also supported by signals delivered through the interleukin-7 receptor (IL-7R) [19, 20].

The immature thymocytes journey through the thymus has also the additional effect of promoting the differentiation of thymic stromal precursors into mature thymic epithelial cells, thus playing an important role in the formation of

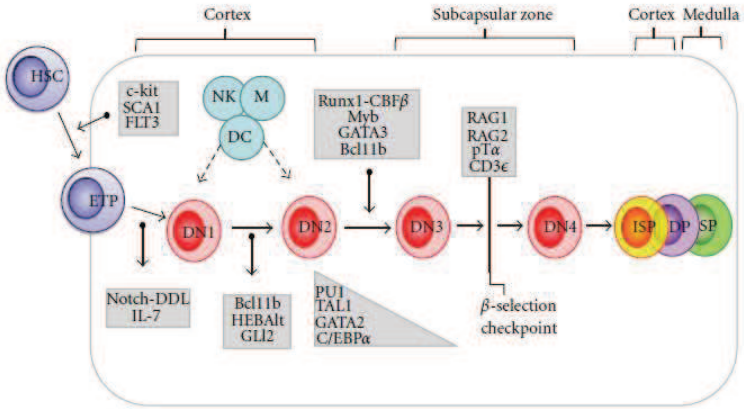

FIGURE 2: Differential gene expression profile, which modulates the discrete stages of the T-cell development. The lymphoid progenitors, entering into thymus and expressing the markers of HSCs, are primed to Notch and IL-7 signaling until DN1 stage. During the transition DN1/DN2, immature thymocytes lose multilineage potential through the downregulation of genes involved in the differentiation towards other cellular lineages, as PU.1, TAL1, GATA-2, and C/EBPα. At the DN2 stage, Myb, GATA-3, HEBalt, GLI-2, and Bcl-11b are upregulated. At the DN3 stage, the genes required for a proper TCR assembly as Rag-1, Rag-2, and pTα are expressed, thus leading to the β-selection. Following β-selection check-point, DN4 cells are fully committed to the TCRαβ$^+$ T-cell lineage.

the thymic microenvironment [21–24]. In particular, thymocytes during the DN1-DN3 stages participate to the differentiation process of TEC precursor cells into cortical TECs (cTECs).

The DN1 cell thymocytes keep the potential to differentiate into B, T, myeloid, NK, and dendritic cells (DCs) [25–27]. The transition to DN2 is characterized by the upregulation of a number of genes involved in the process, including genes needed for rearrangement and/or expression of the pre-TCR signaling complex components (Figure 2) [28]. At this stage, the thymocytes lose the multilineage potential due to silencing of genes involved in the differentiation towards other cellular lineages. Nevertheless, this potential is not completely lost, since cells with the DN2 phenotype can still differentiate into NK cells, DCs, or macrophages under certain circumstances [29, 30].

DN2 stage T cells are fully responsive to IL-7 and SCF due to the high expression of IL-7Rα and c-kit. The DN2 stage is characterized by the upregulation of CD25 molecule (interleukin-2 receptor α, IL-2Rα) and CD90 (Thy-1) [28]. Moreover, the genes which favor the myeloid, NK, and dendritic fate, so-called T-cell antagonists, as PU.1, stem-cell leukemia (SCL also known as TAL1), GATA binding protein-2 (GATA-2), and CCAAT-enhancer binding protein α (C/EBPα) are silenced before that β or γδ selection takes place (Figure 2) [31]. During this phase only a few transcription factors, including the zinc-finger transcription factor, the tumor suppressor factor B-cell lymphoma/leukemia 11b (BCL-11b) [32], basic helix-loop-helix (bHLH) transcription factors alternative (HEBalt) [33], and, more transiently, glioma-associated oncogene 2 (GLI-2), a transcription factor involved in the sonic hedgehog signaling [34], are expressed (Figure 2).

The following DN2 to DN3 stage transition requires the expression of different arrays of genes, as Runt-related transcription factor 1-Core binding factor β (Runx1-CBFβ) complexes, the transcription factor Myb, GATA-3, and Bcl-11b, which allow full TCRβ gene rearrangement in thymocytes, that become competent to undergo β-selection [35–37]. Several important events occur during the DN2/3 transition, as the induction of recombinase activating gene-1 (Rag-1) and Rag-2, the upregulation of pre-Tα (pTα), and the rearrangement of TCRδ and γ. CD3ε and IL-7Rα (CD127) are also upregulated at this phase [38] along with the turn-on of the *lck* tyrosine kinase implicated in the pre-TCR and TCR signaling [39]. At this point, T-cell precursors lose their capability to follow a non-T-cell fate choice [28].

The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage [40]. Thereafter, the E-proteins E2A and HEB play a crucial role in several processes and are required for the progression of the T-cell development. In fact, these proteins are involved in the TCR gene rearrangement [41], in conferring the competence to undergo β-selection, and in the arrest of thymocyte proliferation at the DN3 stage [42].

At the DN3 stage, pre-TCR signaling results in the downregulation of CD25, pTα, Rag-1, and Rag-2, which leads to the appearance of DN4 cells. These cells are fully committed to the αβ T-cell lineage [43, 44]. After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point, the cells become double positive (DP). Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex.

The newly generated DP thymocytes are localized in the cortex and express low levels of the TCRαβ complex. This

DP population consists of T cells with an unselected repertoire [45, 46]. Following that, positive and negative selections take place. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells [47]. When TCR interacts with low-avidity with the peptide-MHC ligands, DP thymocytes receive survival signals. This process, referred to as positive selection, allows "productive" T cells to potentially react to foreign antigens, but not to self-antigens [5]. Lately, positively selected DP thymocytes are ready to differentiate into SP cells, that is, $CD4^+CD8^-$ or $CD4^-CD8^+$ and relocate into the medulla. At this site, newly generated SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator- (AIRE-) expressing mTECs. The cells which are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity [5]. SP thymocytes egress from the thymus as recent thymic emigrants (RTEs), naïve cells expressing the CD62 ligand (CD62L), also known as lymphocyte- (L-) selectin, CD69, and the CD45RA isoform. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [48–50].

3. Pathogenetic Mechanisms of T-Cell Defects

Most of the pathogenic mechanisms underlying primary T-cell disorders are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process requires a crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to alteration of the stromal component of the thymus.

T-cell disorders include a wide spectrum of disorders that affect T-cell development and/or function. The severity of the T-cell defect varies a lot ranging from the syndrome of severe combined immunodeficiency (SCID), characterized by a complete absence of T-cell functions to combined immunodeficiency disorders, in which there are a low number of T cells whose function is not adequate [51].

SCIDs comprise a heterogeneous group of monogenic disorders characterized by a virtual lack of functional peripheral T cells. To date, more than 20 different genetic defects involved in the pathogenesis of SCID in humans have been identified [52, 53]. Typically, patients with SCID show a severe defect in T-cell differentiation and a direct or indirect impairment of B-cell development and function. On the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical phenotypes, SCIDs have been till now classified according to the presence or absence of T, B, and NK cells (Table 1). Impaired survival of lymphocyte precursors is observed in reticular dysgenesis (RD) and in adenosine deaminase (ADA) deficiency. In RD the mutations of the adenylate kinase 2 gene (AK2) result in increased apoptosis of myeloid and lymphoid precursors. As a consequence, patients with RD show marked lymphopenia and neutropenia [54, 55]. ADA deficiency is characterized by the accumulation of high intracellular levels of toxic phosphorylated metabolites of adenosine and deoxyadenosine that cause apoptosis of lymphoid precursors in the bone marrow and thymus [56, 57].

Table 1: SCIDs classification. SCIDs have been so far classified according to the presence or absence of T, B, and NK cells, as a consequence of different molecular defects.

Lymphocyte phenotype	Gene defect	Form of SCID
$T^-B^-NK^-$	Adenylate kinase	Reticular dysgenesis
	Adenosine deaminase	ADA deficiency
$T^-B^+NK^-$	IL-2Rγ	SCID-X1
	Jak3	SCID-AR
$T^-B^+NK^+$	IL-7Rα	IL-7Rα deficiency
$T^-B^-NK^+$	Rag-1 or Rag-2 artemis	Omenn syndrome Artemis deficiency

The majority of SCIDs in human subjects derive from alterations of the cytokine-mediated signaling apparatus. SCID-X1 represents the most common form of SCID and is caused by mutations of the IL-2 receptor γ gene (IL-2Rγ), which encodes for the common γ-chain (γ-c) shared by cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Patients usually have few or no T and NK cells but a normal or elevated number of B cells which fail to produce immunoglobulins normally [58]. γ-c also plays effects on cell cycle control and participates to the growth of tumoral cells, as well [59, 60]. Defects of JAK3, an intracellular tyrosine kinase physically and functionally coupled to γ-c, result in a syndrome whose immunologic phenotype is undistinguishable from that of SCID-X1 [61]. Mutations in the gene encoding for the α-chain of the IL-7R abrogate T lymphocyte development but leave B and NK cell development intact [62]. Mutations in critical genes needed for the expression of pre-T-cell receptor, as Rag-1 and Rag-2, result in a functional inability to form antigen receptors through genetic recombination, compromising the production of functional T cells. These proteins recognize recombination signal sequences and introduce a DNA double-stranded break, permitting V, D, and J gene rearrangements [63, 64]. Lymphocyte phenotype differs from those of patients with SCID caused by γ-c, Janus kinase-3 (Jak-3), IL-7Rα, or ADA deficiencies in that they lack both B and T lymphocytes since pre-TCR and pre-B-cell receptor (BCR) share similar molecular mechanisms requiring Rag-1 and 2 expression [65]. Defects of pre-TCR and pre-BCR expression might also reflect mutations in genes that encode proteins involved in nonhomologous end-joining (NHEJ) and DNA repair and, in particular, Artemis, DNA protein-kinase catalytic subunit (DNA-PKcs), Cernunnos/XLF, and DNA ligase IV [65–69]. In all these diseases, the generation of both T and B lymphocytes is severely compromised. However, it should be noted that a functional T-cell defect may also be due to infections [70, 71] or during the reconstitution phase following stem cell transplantation [72].

It is noteworthy that all the genes whose alterations lead to the above mentioned forms of SCID selectively impair

the lymphocyte functionality and the ability of these cells to proceed in the developmental pathway. In some cases, as in the case of TrkA mutation [73], the gene has pleiotropic effects resulting in complex multisystemic disorders associated to immunodeficiency.

4. The Murine Model of Athymia: nu/nu Mice

The first example of SCID not primarily related to a hematopoietic cell abnormality but rather to an intrinsic thymic epithelial cell defect is the Nude/SCID phenotype, whose identification contributed to unravel important issues of T-cell ontogeny.

The "nude" phenotype, identified for the first time in mice, results from inactivating mutations in a single gene, originally named winged-helix-nude (whn) and recently known as forkhead box n1 (foxn1) [74]. This murine model was described by Flanagan in 1966, when spontaneously appeared in the Virus Laboratory of Ruchill Hospital in Glasgow (UK) [75–77]. Mice homozygous for the mutation "nude" are hairless, have retarded growth, decreased fertility, and die by 5 months of life for infections. The hairlessness is due to the coiling of the incomplete hair shafts in the dermis caused by the absence of free sulfhydryl groups in the midfollicle region [78]. The "nude" *foxn1* gene does not affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle [79, 80]. The "nude" mice are affected by severe infertility and show small ovaries with low egg counts in the females and no motile sperm in the males [78]. This condition may be the result of changes in hormonal status, as demonstrated by altered serum levels of estradiol, progesterone, and thyroxine [81]. The thymus is absent at birth [82] and there are very few lymphocytes in the thymus dependent areas of the spleen and lymph nodes [83].

Since the abnormal, or even absent, thymus is the hallmark of the "nude" phenotype, these animals develop a profound T-cell deficiency and a severely impaired immune response of either cell-mediated and, indirectly, humoral immunity. In "nude" mice, when the thymus is present in the first days of life, it reveals no normal structure, consisting of a thymic rudiment composed of vesicles or canaliculi delimited by epithelial-like cells, with no trace of lymphoid cells. By the day 14, the "nude" thymus is much smaller compared to the normal [84].

Nu/nu mice show lymphopenia and also low immunoglobulin levels. In the absence of normal T cells originated from the thymus, the development of the antibody forming cells is delayed, although "nude" mice do not lack precursors of antibody forming cells. This indicated that antibody forming cells may mature in the absence of the thymus, albeit at a slower rate [85]. In "nude" mice lymph nodes, the outer cortex with primary nodules and the medullary cords are normal. In the spleen sections from the nu/nu mice, the proportion of red to white pulp is greater than normal and, in some cases, an unusually high number of megakaryocytes are seen in the red pulp. In some spleens, Malpighian follicles, although present, are fewer and smaller than in controls and a depletion of lymphocytes is constant in the close proximity of the central arteriole in the thymus-dependent area. The depletion in the splenic thymus-dependent areas is not as prominent as in the lymph nodes [83]. In man, the prototype of an athymic disorder has long been considered the DiGeorge's Syndrome (DGS), even though main features of athymic murine model and human disease, including immunological signs, are not completely overlapping.

5. The Athymic DiGeorge Syndrome

The DGS, along with velocardiofacial syndrome and conotruncal anomaly face syndrome, is frequently associated to a common heterozygous intrachromosomal deletion in 22q11.2. However, a DGS-like phenotype can have alternative etiologies, including maternal diabetes, fetal alcohol syndrome, and teratogenesis, even though the molecular mechanisms underlying these forms are still unknown [86]. DGS has an estimated incidence of 1 in 4000 live births [87, 88] and, thus, it is the most common microdeletion syndrome in humans and the second most common chromosomal disorder after Down's syndrome. The deletion is due to a meiotic nonallelic homologous recombination between flanking 250 kilobases (kb), mapping in 22q11.2 chromosomal region and consisting in low-copy repeats/segmental duplications in the termed LCR22 [89, 90]. Although most cases of DGS occur as *de novo* deletions, approximately 5% of cases are inherited as an autosomal dominant trait [91–93]. In the 90% of patients, a hemizygous 3 Mb deletion, containing about 30 genes [89, 90, 94, 95], is found, whereas approximately 8% of patients carry a smaller deletion of 1.5 Mb, encompassing 24 genes [96], even though no difference in the clinical presentation is appreciable in the smaller deletion [86].

The main features of this syndrome are mild facial dysmorphism, submucous cleft palate, velopharyngeal insufficiency, speech delay, recurrent infections, variable immunodeficiency secondary to thymic aplasia or hypoplasia, and cardiac anomalies [97, 98]. Most of the patients have learning disabilities and behavioral disorders, including schizophrenia in some cases [99–102]. Children with the DGS, according to the aplasia or hypoplasia of the thymus, are classified as complete or partial DGS. The "complete" form represents a small percentage of patients, accounting to the 0.5% of all patients. These patients show a severe combined immunodeficiency phenotype with near absent T lymphocytes. The majority of patients have a "partial" phenotype and an immune defect usually manifesting as mild to moderate T lymphocytopenia. The T-cell proliferation is usually normal or in very few cases low normal. These patients have been reported to have a moderate increase of the number of infections than predicted on the basis of the immunological impairment, suggesting that anatomical defects, gastroesophageal reflux, allergies, cardiac disease, and poor nutrition may also contribute to recurrent infections [103]. It should be underlined that never "partial" DGS patients have severe infections as reported in SCID and, moreover, T-cell proliferation is usually normal. A moderate CD4 lymphocytopenia with low to normal CD8 T lymphocytes is usually found. An age-related decrease of T lymphocytes is also seen in DGS patients. TCR

repertoire analysis in 22q11.2 deletion patients has shown significant oligoclonal peaks and Vβ family dropouts when compared to controls. In a study of nine patients with a negative infectious history, a decreased diversity in $CD4^+$ and $CD8^+$ TCR repertoire, using both flow cytometric and third complementarity determining region (CDR3 spectratyping) fragment analysis, has been documented [104]. In another study, the spectratyping showed alterations in the repertoire, which, however, improved over the time [105].

Immune deficiency in these patients seems to be associated to an increased incidence of autoimmune diseases [106–108], in particular cytopenias [109, 110], arthritis [111], and endocrinopathies [112].

The chromosomal region usually deleted contains several genes, which may be candidate of the DGS phenotype. TBX1, which belongs to the family of T-box transcription factors, which share a common DNA binding domain is called "T-box" [113]. A specific role for Tbx1 in DGS and thymus development came out from the peculiar expression pattern in both the third pharyngeal pouch endoderm and the adjacent mesenchyme and not in the neural crest cells [114]. Furthermore, the homozygous loss of *Tbx1* causes thymic hypoplasia, as well [96, 115–117]. Of note, mice heterozygous for a null allele of *Tbx1* demonstrate only a mild phenotype without thymus anomalies [118]. Thus, evidence would suggest, at least in mice, that gene dosage of *Tbx1* is crucial in the pathogenesis of DGS. However, in the same region there are other genes potentially implicated in the pathogenesis of DGS, such as *Crkl*, which encodes an adaptor protein implicated in growth factor and adhesion molecule signaling. Homozygous *Crkl* gene deletion results in multiple defects in neural crest derivatives including aortic arch arteries, thymus, and craniofacial structures [96] and in prenatal death. However, the deletion at the heterozygous state does not cause any clinical sign, thus indicating that a combination of gene alterations is needed for the full expressivity of the phenotype [119].

6. The Human Nude/SCID Phenotype

The human equivalent of the "nude" murine phenotype was first described in two sisters in 1996, after more than 30 years from the initial mouse description and, subsequently, associated to *FOXN1* gene alterations.

The human Nude/SCID is an autosomal recessive disorder [120], whose hallmark is the T-cell immunodeficiency due to the complete absence of the thymus. This immunodeficiency presents in a quite similar fashion to the classical SCID phenotype, thus being more severe than DGS. Along with the severe infections, other features of the syndrome are ectodermal abnormalities, as alopecia and nail dystrophy [121]. Of note, the nail dystrophy can be observed also in subjects carrying the genetic alteration in heterozygosity. The most frequent nail alteration is the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; a canaliform dystrophy associated to a transverse groove of the nail plate (Beau line) may also be found (Figure 3). However, the most specific phenotypic alteration is 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 have also been 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 heterozygosity for this molecular alteration [121].

Interestingly, additional studies have also reported on anomalies of brain structures, suggesting a potential role of this transcription factor in brain embryogenesis, as also suggested by its expression in epithelial cells of the developing choroids plexus, a structure filling the lateral, third, and fourth ventricles. However, the severe neural tube defects, including anencephaly and spina bifida, have been only inconstantly reported, thus probably indicating that the genetic alteration represents a cofactor and is not sufficient *per se* to alter brain embryogenesis. The anomalies of brain structure have been considered potentially responsible for the high rate of mortality *in utero* observed in the geographic area with the high frequency of *FOXN1* alteration [122].

Prenatal alteration of the *FOXN1* gene in humans prevents the development of the T-cell compartment as early as at 16 weeks of gestation [123]. By contrast, stem cells, B, and NK lymphocytes are normal. $CD4^+$ cells are more affected than $CD8^+$ cells, even though the latter are also profoundly reduced. No $CD4^+CD45RA^+$ naive cells can be usually found [123]. CD8 cells coexpressing CD3 are very scarce and a few $CD3^+CD8^+CD45RA^+$ naïve cells can be detected [123]. Overall, a substantial reduction of T cells bearing TCR$\alpha\beta$, but not of lymphocytes expressing TCR$\gamma\delta$, is observed [123]. TCR gene rearrangement, although altered, occurs to some extent, suggesting the possibility of an extrathymic and FOXN1-independent site of differentiation. However, it should be emphasized that these few T cells, which escape the blockage, are unable to sustain a productive immune response into the periphery.

Taken together, the data so far available underline the crucial role of FOXN1 in the early prenatal stages of T-cell ontogeny in humans [123].

7. Role of FOXN1 in Immune System

FOXN1 belongs to the forkhead-box gene family that comprises a diverse group of "winged helix" transcription factors implicated in a variety of cellular processes: development, metabolism, cancer, and aging [124]. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions. While during embryogenesis *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, later, its expression is confined to skin and thymus epithelia, where FOXN1 is absolutely required for the normal differentiation of hair follicles and TECs.

FOXN1 gene, spanning about 30 kb [125, 126], is an epithelial cell-autonomous gene and is highly conserved in sequence and function in rodents and humans. Interestingly,

(a) (b) (c)

FIGURE 3: Nail dystrophy patterns in subjects carrying heterozygous mutations in FOXN1 gene: (a) koilonychias, (b) canaliform dystrophy, and (c) leukonychia.

an extensive screening of cDNA clones obtained from skin cells revealed the presence of two different noncoding first exons [126], 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 [125]. The alternative usage of the exon 1a or 1b seems to direct the tissue specificity [126], in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin.

The molecular mechanisms by which *FOXN1* expression and activity are regulated are only incompletely understood. It is suggested that FOXN1 might, subsequently, upregulate the expression of fibroblast growth factor (FGF) receptors, which in turn modulate the thymic stroma differentiation and thymopoiesis [127]. *In vitro* exposure of thymic epithelial cells to some Wnt proteins is sufficient to upregulate FOXN1 protein expression in both an endocrine and paracrine fashion [128]. Wnts belong to a large family of secreted glycoproteins that have important roles in cell-fate specification [127].

The prenatal thymus development, the maintenance of a proper thymic microenvironment, and the efficient T-cell production require an appropriate crass-talk between thymocytes and thymic stromal cells [12]. Postnatally, the thymic involution results in dramatically reduced T-cell generation in an age-dependent fashion [129].

Indeed, recent evidence has implicated both TEC- and hematopoietic stem cell- (HSC-) intrinsic defects in involution of the organ [130–133]. *Foxn1* is expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation [75, 134, 135]. *Foxn1* exerts an important role [136] in inducing both cortical and medullary differentiation [137, 138]. Although foxn1 has long been studied, most of the studies thus far available are restricted to fetal differentiation process, while its postnatal role in the mature thymus still remains to be fully elucidated.

However, it is largely unknown whether the role of foxn1 in the thymus and skin is identical. One important difference is that foxn1 is involved in morphogenesis of the three-dimensional thymic microstructure, which is important for the functionality of the thymus [139]. Moreover, the differentiation of the immature epithelial cells into functional cTECs and mTECs is foxn1-dependent. In particular, foxn1 mainly regulates TEC patterning in the fetal stage [140] and TEC homeostasis in the postnatal thymus [141]. TECs are implicated in either thymus organogenesis or in most stages of maturation of thymocytes [142, 143]. The inborn null mutation in *foxn1* [76] causes a differentiation failure in TECs thereby halting thymic development at a rudimentary stage. The thymic lobar architecture is still present but the epithelial cells lack the ability to induce the entrance of hematopoietic precursor cells (HPCs) into the epithelial cluster and thus preclude the generation of thymocytes [144]. These results argue strongly for a failure in thymocytes-epithelial crosstalk, thus, explaining the blockage of thymic lymphopoiesis [75, 136]. The organ is, therefore, an alymphoid two-dimensional (2D) rudiment with a cystic structure [72, 82, 120, 123].

Because of 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 HPCs. This finding would imply a role for skin as a primary lymphoid organ [145].

8. Conclusion and Future Research

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs. These disorders include a wide spectrum of diseases that affect T-cell development and/or function. The pathogenic mechanisms are mostly related to molecular alterations of genes selectively expressed in hematopoietic cells. However, they can also be due to alterations of the stromal component of the thymus, which is the primary lymphoid organ that supports T-cell differentiation and repertoire selection. In this organ, the dynamic relocation in multiple architectural structures requires the crosstalk between thymocytes and thymic microenvironment. The Nude/SCID syndrome results from inactivating mutations in the gene encoding the *FOXN1* transcriptional factor selectively expressed in skin and thymic epithelia. In mice and humans its alteration leads to thymic agenesia and severe T-cell deficiency. The Nude/SCID immunodeficiency is much more severe than DGS, indicating that the *FOXN1* expression is absolutely required for an efficient production of mature T cells. The studies on the human Nude/SCID

phenotype greatly contributed to unravel important issues of the T-cell ontogeny and, in the near future, may help define potential extrathymic and thymus-independent sites of differentiation in man.

References

[1] H. Chapel, R. Geha, and F. Rosen, "Primary immunodeficiency diseases: an update," *Clinical and Experimental Immunology*, vol. 132, no. 1, pp. 9–15, 2003.

[2] J. D. Edgar, "T cell immunodeficiency," *Journal of Clinical Pathology*, vol. 61, no. 9, pp. 988–993, 2008.

[3] M. J. Bevan, "In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells," *Nature*, vol. 269, no. 5627, pp. 417–418, 1977.

[4] J. F. Miller, "Immunological function of the thymus," *The Lancet*, vol. 2, no. 7205, pp. 748–749, 1961.

[5] Y. Takahama, "Journey through the thymus: stromal guides for T-cell development and selection," *Nature Reviews Immunology*, vol. 6, no. 2, pp. 127–135, 2006.

[6] N. R. Manley, "Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation," *Seminars in Immunology*, vol. 12, no. 5, pp. 421–428, 2000.

[7] T. Boehm, C. C. Bleul, and M. Schorpp, "Genetic dissection of thymus development in mouse and zebrafish," *Immunological Reviews*, vol. 195, no. 1, pp. 15–27, 2003.

[8] B. F. Haynes and C. S. Heinly, "Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment," *Journal of Experimental Medicine*, vol. 181, no. 4, pp. 1445–1458, 1995.

[9] J. J. Owen and M. A. Ritter, "Tissue interaction in the development of thymus lymphocytes," *Journal of Experimental Medicine*, vol. 129, no. 2, pp. 431–442, 1969.

[10] C. C. Bleul and T. Boehm, "Chemokines define distinct microenvironments in the developing thymus," *European Journal of Immunology*, vol. 30, no. 12, pp. 3371–3379, 2000.

[11] C. Liu, T. Ueno, S. Kuse et al., "The role of CCL21 in recruitment of T-precursor cells to fetal thymi," *Blood*, vol. 105, no. 1, pp. 31–39, 2005.

[12] D. H. Gray, T. Ueno, A. P. Chidgey et al., "Controlling the thymic microenvironment," *Current Opinion in Immunology*, vol. 17, no. 2, pp. 137–143, 2005.

[13] M. A. Wurbel, M. Malissen, D. Guy-Grand et al., "Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor $\gamma\delta^+$ gut intraepithelial lymphocytes," *Blood*, vol. 98, no. 9, pp. 2626–2632, 2001.

[14] T. Ara, M. Itoi, K. Kawabata et al., "A role of CXC chemokine ligand 12/stromal cell-derived factor-1/pre-B cell growth stimulating factor and its receptor CXCR4 in fetal and adult T cell development in vivo," *Journal of Immunology*, vol. 170, no. 9, pp. 4649–4655, 2003.

[15] D. I. Godfrey and A. Zlotnik, "Control points in early T-cell development," *Immunology Today*, vol. 14, no. 11, pp. 547–553, 1993.

[16] H. T. Petrie and J. C. Zúñiga-Pflücker, "Zoned out: functional mapping of stromal signaling microenvironments in the thymus," *Annual Review of Immunology*, vol. 25, pp. 649–679, 2007.

[17] F. Radtke, A. Wilson, G. Stark et al., "Deficient T cell fate specification in mice with an induced inactivation of Notch1," *Immunity*, vol. 10, no. 5, pp. 547–558, 1999.

[18] J. C. Zuniga-Pflucker, "T-cell development made simple," *Nature Reviews Immunology*, vol. 4, no. 1, pp. 67–72, 2004.

[19] J. J. Peschon, P. J. Morrissey, K. H. Grabstein et al., "Early lymphocyte expansion is severely impaired in interleukin 7 receptor- deficient mice," *Journal of Experimental Medicine*, vol. 180, no. 5, pp. 1955–1960, 1994.

[20] U. von Freeden-Jeffry, P. Vieira, L. A. Lucian, T. McNeil, S. E. Burdach, and R. Murray, "Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine," *Journal of Experimental Medicine*, vol. 181, no. 4, pp. 1519–1526, 1995.

[21] G. A. Hšllander, B. Wang, A. Nichogiannopoulou et al., "Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes," *Nature*, vol. 373, no. 6512, pp. 350–353, 1995.

[22] W. van Ewijk, G. Hollander, C. Terhorst, and B. Wang, "Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets," *Development*, vol. 127, no. 8, pp. 1583–1591, 2000.

[23] D. B. Klug, C. Carter, I. B. Gimenez-Conti, and E. R. Richie, "Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus," *Journal of Immunology*, vol. 169, no. 6, pp. 2842–2845, 2002.

[24] D. B. Klug, C. Carter, E. Crouch, D. Roop, C. J. Conti, and E. R. Richie, "Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11822–11827, 1998.

[25] K. Shortman and L. Wu, "Early T lymphocyte progenitors," *Annual Review of Immunology*, vol. 14, pp. 29–47, 1996.

[26] C. Y. Wu, R. R. Warrier, D. M. Carvajal et al., "Biological function and distribution of human interleukin-12 receptor β chain," *European Journal of Immunology*, vol. 26, no. 2, pp. 345–350, 1996.

[27] T. A. Moore and A. Zlotnik, "T-cell lineage commitment and cytokine responses of thymic progenitors," *Blood*, vol. 86, no. 5, pp. 1850–1860, 1995.

[28] G. Anderson, W. E. Jenkinson, T. Jones et al., "Establishment and functioning of intrathymic microenvironments," *Immunological Reviews*, vol. 209, no. 1, pp. 10–27, 2006.

[29] C. K. Lee, J. K. Kim, Y. Kim et al., "Generation of macrophages from early T progenitors in vitro," *Journal of Immunology*, vol. 166, no. 10, pp. 5964–5969, 2001.

[30] T. M. Schmitt, M. Ciofani, H. T. Petrie, and J. C. Zúñiga-Pflücker, "Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions," *Journal of Experimental Medicine*, vol. 200, no. 4, pp. 469–479, 2004.

[31] E. V. Rothenberg, J. E. Moore, and M. A. Yui, "Launching the T-cell-lineage developmental programme," *Nature Reviews Immunology*, vol. 8, no. 1, pp. 9–21, 2008.

[32] Y. Wakabayashi, H. Watanabe, J. Inoue et al., "Bcl11b is required for differentiation and survival of $\alpha\beta$ T lymphocytes," *Nature Immunology*, vol. 4, no. 6, pp. 533–539, 2003.

[33] C. C. Tydell, E. S. David-Fung, J. E. Moore, L. Rowen, T. Taghon, and E. V. Rothenberg, "Molecular dissection of prethymic progenitor entry into the T lymphocyte developmental pathway," *Journal of Immunology*, vol. 179, no. 1, pp. 421–438, 2007.

[34] A. El Andaloussi, S. Graves, F. Meng, M. Mandal, M. Mashayekhi, and I. Aifantis, "Hedgehog signaling controls thymocyte progenitor homeostasis and differentiation in the thymus," *Nature Immunology*, vol. 7, no. 4, pp. 418–426, 2006.

[35] L. Talebian, Z. Li, Y. Guo et al., "T-lymphoid, megakaryocyte, and granulocyte development are sensitive to decreases in CBFβ dosage," *Blood*, vol. 109, no. 1, pp. 11–21, 2007.

[36] T. P. Bender, C. S. Kremer, M. Kraus, T. Buch, and K. Rajewsky, "Critical functions for c-Myb at three checkpoints during thymocyte development," *Nature Immunology*, vol. 5, no. 7, pp. 721–729, 2004.

[37] J. Inoue, T. Kanefuji, K. Okazuka, H. Watanabe, Y. Mishima, and R. Kominami, "Expression of TCR$\alpha\beta$ partly rescues developmental arrest and apoptosis of $\alpha\beta$ T cells in Bcl11b$^{-/-}$ mice," *Journal of Immunology*, vol. 176, no. 10, pp. 5871–5879, 2006.

[38] T. N. Taghon, E. S. David, J. C. Zúñiga-Pflücker, and E. V. Rothenberg, "Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling," *Genes and Development*, vol. 19, no. 8, pp. 965–978, 2005.

[39] C. Shimizu, H. Kawamoto, M. Yamashita et al., "Progression of T cell lineage restriction in the earliest subpopulation of murine adult thymus visualized by the expression of lck proximal promoter activity," *International Immunology*, vol. 13, no. 1, pp. 105–117, 2001.

[40] H. von Boehmer and H. J. Fehling, "Structure and function of the pre-T cell receptor," *Annual Review of Immunology*, vol. 15, pp. 433–452, 1997.

[41] C. Murre, "Helix-loop-helix proteins and lymphocyte development," *Nature Immunology*, vol. 6, no. 11, pp. 1079–1086, 2005.

[42] J. Wojciechowski, A. Lai, M. Kondo, and Y. Zhuang, "E2A and HEB are required to block thymocyte proliferation prior to pre-TCR expression," *Journal of Immunology*, vol. 178, no. 9, pp. 5717–5726, 2007.

[43] J. C. Zúñiga-Pflücker and M. J. Lenardo, "Regulation of thymocyte development from immature progenitors," *Current Opinion in Immunology*, vol. 8, no. 2, pp. 215–224, 1996.

[44] H. J. Fehling and H. von Boehmer, "Early $\alpha\beta$ T cell development in the thymus of normal and genetically altered mice," *Current Opinion in Immunology*, vol. 9, no. 2, pp. 263–275, 1997.

[45] P. Kisielow, H. S. Teh, H. Bluthmann, and H. von Boehmer, "Positive selection of antigen-specific T cells in thymus by restricting MHC molecules," *Nature*, vol. 335, no. 6192, pp. 730–733, 1988.

[46] S. C. Jameson, K. A. Hogquist, and M. J. Bevan, "Positive selection of thymocytes," *Annual Review of Immunology*, vol. 13, pp. 93–126, 1995.

[47] P. Bousso, N. R. Bhakta, R. S. Lewis, and E. Robey, "Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy," *Science*, vol. 296, no. 5574, pp. 1876–1880, 2002.

[48] R. A. Reichert, I. L. Weissman, and E. C. Butcher, "Phenotypic analysis of thymocytes that express homing receptors for peripheral lymph nodes," *Journal of Immunology*, vol. 136, no. 10, pp. 3521–3528, 1986.

[49] A. Bendelac, P. Matzinger, R. A. Seder, W. E. Paul, and R. H. Schwartz, "Activation events during thymic selection," *Journal of Experimental Medicine*, vol. 175, no. 3, pp. 731–742, 1992.

[50] F. Ramsdell, M. Jenkins, Q. Dinh, and B. J. Fowlkes, "The majority of CD4$^+$8$^-$ thymocytes are functionally immature," *Journal of Immunology*, vol. 147, no. 6, pp. 1779–1785, 1991.

[51] R. H. Buckley, "Primary cellular immunodeficiencies," *Journal of Allergy and Clinical Immunology*, vol. 109, no. 5, pp. 747–757, 2002.

[52] R. H. Buckley, "Advances in the understanding and treatment of human severe combined immunodeficiency," *Immunologic Research*, vol. 22, no. 2-3, pp. 237–251, 2000.

[53] A. Fischer, "Human primary immunodeficiency diseases: a perspective," *Nature Immunology*, vol. 5, no. 1, pp. 23–30, 2004.

[54] U. Pannicke, M. Hönig, I. Hess et al., "Reticular dysgenesis (aleukocytosis) is caused by mutations in the gene encoding mitochondrial adenylate kinase 2," *Nature Genetics*, vol. 41, no. 1, pp. 101–105, 2009.

[55] C. Lagresle-Peyrou, E. M. Six, C. Picard et al., "Human adenylate kinase 2 deficiency causes a profound hematopoietic defect associated with sensorineural deafness," *Nature Genetics*, vol. 41, no. 1, pp. 106–111, 2009.

[56] W. L. Nyhan, "Disorders of purine and pyrimidine metabolism," *Molecular Genetics and Metabolism*, vol. 86, no. 1-2, pp. 25–33, 2005.

[57] F. Malacarne, T. Benicchi, L. D. Notarangelo et al., "Reduced thymic output, increased spontaneous apoptosis and oligoclonal B cells in polyethylene glycol-adenosine deaminase-treated patients," *European Journal of Immunology*, vol. 35, no. 11, pp. 3376–3386, 2005.

[58] H. White, A. Thrasher, P. Veys, C. Kinnon, and H. B. Gaspar, "Intrinsic defects of B cell function in X-linked severe combined immunodeficiency," *European Journal of Immunology*, vol. 30, no. 3, pp. 732–737, 2000.

[59] M. Adriani, C. Garbi, G. Amodio et al., "Functional interaction of common γ-chain and growth hormone receptor signaling apparatus," *Journal of Immunology*, vol. 177, no. 10, pp. 6889–6895, 2006.

[60] S. Amorosi, I. Russo, G. Amodio et al., "The cellular amount of the common γ-chain influences spontaneous or induced cell proliferation," *Journal of Immunology*, vol. 182, no. 5, pp. 3304–3309, 2009.

[61] P. Macchi, A. Villa, S. Giliani et al., "Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID)," *Nature*, vol. 377, no. 6544, pp. 65–68, 1995.

[62] A. Puel, S. F. Ziegler, R. H. Buckley, and W. J. Leonard, "Defective IL7R expression in T$^-$B$^+$NK$^+$ severe combined immunodeficiency," *Nature Genetics*, vol. 20, no. 4, pp. 394–397, 1998.

[63] K. Schwarz, G. H. Gauss, L. Ludwig et al., "RAG mutations in human B cell-negative SCID," *Science*, vol. 274, no. 5284, pp. 97–99, 1996.

[64] B. Corneo, D. Moshous, T. Güngör et al., "Identical mutations in RAG1 or RAG2 genes leading to defective V(D)J recombinase activity can cause either T-B-severe combined immune deficiency or Omenn syndrome," *Blood*, vol. 97, no. 9, pp. 2772–2776, 2001.

[65] P. Revy, D. Buck, F. le Deist, and J. P. de Villartay, "The repair of DNA damages/modifications during the maturation of the immune system: lessons from human primary immunodeficiency disorders and animal models," *Advances in Immunology*, vol. 87, pp. 237–295, 2005.

[66] M. van der Burg, J. J. van Dongen, and D. C. van Gent, "DNA-PKcs deficiency in human: long predicted, finally found," *Current Opinion in Allergy and Clinical Immunology*, vol. 9, no. 6, pp. 503–509, 2009.

[67] M. van der Burg, C. M. Weemaes, F. Preijers et al., "B-cell recovery after stem cell transplantation of Artemis-deficient SCID requires elimination of autologous bone marrow precursor-B-cells," *Haematologica*, vol. 91, no. 12, pp. 1705–1709, 2006.

[68] D. Buck, L. Malivert, R. de Chasseval et al., "Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly," *Cell*, vol. 124, no. 2, pp. 287–299, 2006.

[69] A. Fischer, F. le Deist, S. Hacein-Bey-Abina et al., "Severe combined immunodeficiency. A model disease for molecular immunology and therapy," *Immunological Reviews*, vol. 203, pp. 98–109, 2005.

[70] X. X. J. Gu, F. Y. Yue, C. M. Kovacs, and M. A. Ostrowski, "The role of cytokines which signal through the common γ chain cytokine receptor in the reversal of HIV specific $CD4^+$ and $CD8^+$ T cell anergy," *PLoS One*, vol. 2, no. 3, article e300, 2007.

[71] C. Pignata, M. Fiore, S. de Filippo, M. Cavalcanti, L. Gaetaniello, and I. Scotese, "Apoptosis as a mechanism of peripheral blood mononuclear cell death following measles and varicella-zoster virus infections in children," *Pediatric Research*, vol. 43, no. 1, pp. 77–83, 1998.

[72] C. Pignata, L. Gaetaniello, A. M. Masci et al., "Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation," *Blood*, vol. 97, no. 4, pp. 880–885, 2001.

[73] E. Toscano, R. della Casa, S. Mardy et al., "Multisystem involvement in congenital insensitivity to pain with anhidrosis (CIPA), a nerve growth factor receptor (Trk A)-related disorder," *Neuropediatrics*, vol. 31, no. 1, pp. 39–41, 2000.

[74] K. H. Kaestner, W. Knöchel, and D. E. Martínez, "Unified nomenclature for the winged helix/forkhead transcription factors," *Genes and Development*, vol. 14, no. 2, pp. 142–146, 2000.

[75] M. Nehls, B. Kyewski, M. Messerle et al., "Two genetically separable steps in the differentiation of thymic epithelium," *Science*, vol. 272, no. 5263, pp. 886–889, 1996.

[76] M. Nehls, D. Pfeifer, M. Schorpp, H. Hedrich, and T. Boehm, "New member of the winged-helix protein family disrupted in mouse and rat nude mutations," *Nature*, vol. 372, no. 6501, pp. 103–107, 1994.

[77] J. A. Segre, J. L. Nemhauser, B. A. Taylor, J. H. Nadeau, and E. S. Lander, "Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat," *Genomics*, vol. 28, no. 3, pp. 549–559, 1995.

[78] S. P. Flanagan, "'Nude', a new hairless gene with pleiotropic effects in the mouse," *Genetical Research*, vol. 8, no. 3, pp. 295–309, 1966.

[79] J. L. Brissette, J. Li, J. Kamimura, D. Lee, and G. P. Dotto, "The product of the mouse nude locus, whn, regulates the balance between epithelial cell growth and differentiation," *Genes and Development*, vol. 10, no. 17, pp. 2212–2221, 1996.

[80] D. Lee, D. M. Prowse, and J. L. Brissette, "Association between mouse nude gene expression and the initiation of epithelial terminal differentiation," *Developmental Biology*, vol. 208, no. 2, pp. 362–374, 1999.

[81] P. Köpf-Maier and V. F. Mboneko, "Anomalies in the hormonal status of athymic nude mice," *Journal of Cancer Research and Clinical Oncology*, vol. 116, no. 3, pp. 229–231, 1990.

[82] E. M. Pantelouris, "Absence of thymus in a mouse mutant," *Nature*, vol. 217, no. 5126, pp. 370–371, 1968.

[83] M. A. B. de Sousa, D. M. V. Parrott, and E. M. Pantelouris, "The lymphoid tissues in mice with congenital aplasia of the thymus," *Clinical and Experimental Immunology*, vol. 4, no. 6, pp. 637–644, 1969.

[84] E. M. Pantelouris and J. Hair, "Thymus dysgenesis in nude (nu/nu) mice," *Journal of Embryology and Experimental Morphology*, vol. 24, no. 3, pp. 615–623, 1970.

[85] H. H. Wortis, "Immunological responses of 'nude' mice," *Clinical and Experimental Immunology*, vol. 8, no. 2, pp. 305–317, 1971.

[86] R. Paylor and E. Lindsay, "Mouse models of 22q11 deletion syndrome," *Biological Psychiatry*, vol. 59, no. 12, pp. 1172–1179, 2006.

[87] L. D. Botto, K. May, P. M. Fernhoff et al., "A population-based study of the 22q11.2 Deletion: phenotype, incidence, and contribution to major birth defects in the population," *Pediatrics*, vol. 112, no. 1, pp. 101–107, 2003.

[88] K. Devriendt, J. P. Fryns, G. Mortier, M. N. van Thienen, and K. Keymolen, "The annual incidence of DiGeorge/velocardiofacial syndrome," *Journal of Medical Genetics*, vol. 35, no. 9, pp. 789–790, 1998.

[89] L. Edelmann, R. K. Pandita, and B. E. Morrow, "Low-copy repeats mediate the common 3-Mb deletion in patients with velo- cardio-facial syndrome," *American Journal of Human Genetics*, vol. 64, no. 4, pp. 1076–1086, 1999.

[90] T. H. Shaikh, H. Kurahashi, S. C. Saitta et al., "Chromosome 22-specific low copy repeats and the 22q11.2 deletion syndrome: genomic organization and deletion endpoint analysis," *Human Molecular Genetics*, vol. 9, no. 4, pp. 489–501, 2000.

[91] A. Swillen, K. Devriendt, G. Vantrappen et al., "Familial deletions of chromosome 22q11: the Leuven experience," *American Journal of Medical Genetics*, vol. 80, no. 5, pp. 531–532, 1998.

[92] M. A. Williams, R. J. Shprintzen, and R. B. Goldberg, "Male-to-male transmission of the velo-cardio-facial syndrome: a case report and review of 60 cases," *Journal of Craniofacial Genetics and Developmental Biology*, vol. 5, no. 2, pp. 175–180, 1985.

[93] M. C. Digilio, B. Marino, A. Giannotti, and B. Dallapiccola, "Familial deletions of chromosome 22q11," *American Journal of Medical Genetics*, vol. 73, no. 1, pp. 95–96, 1997.

[94] B. Morrow, R. Goldberg, C. Carlson et al., "Molecular definition of the 22q11 deletions in velo-cardio-facial syndrome," *American Journal of Human Genetics*, vol. 56, no. 6, pp. 1391–1403, 1995.

[95] E. A. Lindsay, R. Goldberg, V. Jurecic et al., "Velo-cardio-facial syndrome: frequency and extent of 22q11 deletions," *American Journal of Medical Genetics*, vol. 57, no. 3, pp. 514–522, 1995.

[96] E. A. Lindsay, "Chromosomal microdeletions: dissecting del22q11 syndrome," *Nature Reviews Genetics*, vol. 2, no. 11, pp. 858–868, 2001.

[97] A. K. Ryan, J. A. Goodship, D. I. Wilson et al., "Spectrum of clinical features associated with interstitial chromosome 22q11 deletions: a European collaborative study," *Journal of Medical Genetics*, vol. 34, no. 10, pp. 798–804, 1997.

[98] R. J. Shprintzen, "Velo-cardio-facial syndrome: 30 years of study," *Developmental Disabilities Research Reviews*, vol. 14, no. 1, pp. 3–10, 2008.

[99] E. W. Chow, A. S. Bassett, and R. Weksberg, "Velo-cardio-facial syndrome and psychotic disorders: implications for psychiatric genetics," *American Journal of Medical Genetics*, vol. 54, no. 2, pp. 107–112, 1994.

[100] K. C. Murphy and M. J. Owen, "Velo-cardio-facial syndrome: a model for understanding the genetics and pathogenesis of schizophrenia," *British Journal of Psychiatry*, vol. 179, pp. 397–402, 2001.

[101] L. J. Evers, C. E. de Die-Smulders, E. E. Smeets, M. G. Clerkx, and L. M. Curfs, "The velo-cardio-facial syndrome: the spectrum of psychiatric problems and cognitive deterioration at adult age," *Genetic Counseling*, vol. 20, no. 4, pp. 307–315, 2009.

[102] R. J. Shprintzen, "Velo-cardio-facial syndrome: a distinctive behavioral phenotype," *Mental Retardation and Developmental Disabilities Research Reviews*, vol. 6, no. 2, pp. 142–147, 2000.

[103] L. J. Kobrynski and K. E. Sullivan, "Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes," *The Lancet*, vol. 370, no. 9596, pp. 1443–1452, 2007.

[104] M. Pierdominici, F. Mazzetta, E. Caprini et al., "Biased T-cell receptor repertoires in patients with chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome)," *Clinical and Experimental Immunology*, vol. 132, no. 2, pp. 323–331, 2003.

[105] C. Cancrini, M. L. Romiti, A. Finocchi et al., "Post-natal ontogenesis of the T-cell receptor CD4 and CD8 Vβ repertoire and immune function in children with DiGeorge syndrome," *Journal of Clinical Immunology*, vol. 25, no. 3, pp. 265–274, 2005.

[106] P. D. Arkwright, M. Abinun, and A. J. Cant, "Autoimmunity in human primary immunodeficiency diseases," *Blood*, vol. 99, no. 8, pp. 2694–2702, 2002.

[107] A. Etzioni and S. Pollack, "Autoimmune phenomena in DiGeorge syndrome," *Israel Journal of Medical Sciences*, vol. 30, no. 11, p. 853, 1994.

[108] A. F. Jawad, D. M. McDonald-McGinn, E. Zackai, and K. E. Sullivan, "Immunologic features of chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome)," *Journal of Pediatrics*, vol. 139, no. 5, pp. 715–723, 2001.

[109] J. K. Davies, P. Telfer, J. D. Cavenagh, N. Foot, and M. Neat, "Autoimmune cytopenias in the 22q11.2 deletion syndrome," *Clinical and Laboratory Haematology*, vol. 25, no. 3, pp. 195–197, 2003.

[110] O. Sakamoto, M. Imaizumi, A. Suzuki et al., "Refractory autoimmune hemolytic anemia in a patient with chromosome 22q11.2 deletion syndrome," *Pediatrics International*, vol. 46, no. 5, pp. 612–614, 2004.

[111] K. E. Sullivan, D. M. McDonald-McGinn, D. A. Driscoll et al., "Juvenile rheumatoid arthritis-like polyarthritis in chromosome 22q11.2 deletion syndrome (DiGeorge anomalad/velocardiofacial syndrome/conotruncal anomaly face syndrome)," *Arthritis & Rheumatism*, vol. 40, no. 3, pp. 430–436, 1997.

[112] J. J. Brown, V. Datta, M. J. Browning, and P. G. Swift, "Graves' disease in DiGeorge syndrome: patient report with a review of endocrine autoimmunity associated with 22q11.2 deletion," *Journal of Pediatric Endocrinology and Metabolism*, vol. 17, no. 11, pp. 1575–1579, 2004.

[113] J. Smith, "T-box genes: what they do and how they do it," *Trends in Genetics*, vol. 15, no. 4, pp. 154–158, 1999.

[114] G. Hollander, J. Gill, S. Zuklys, N. Iwanami, C. Liu, and Y. Takahama, "Cellular and molecular events during early thymus development," *Immunological Reviews*, vol. 209, no. 1, pp. 28–46, 2006.

[115] E. A. Lindsay, A. Botta, V. Jurecic et al., "Congenital heart disease in mice deficient for the DiGeorge syndrome region," *Nature*, vol. 401, no. 6751, pp. 379–383, 1999.

[116] S. Merscher, B. Funke, J. A. Epstein et al., "TBX1 is responsible for cardiovascular defects in velo-cardio-facial/DiGeorge syndrome," *Cell*, vol. 104, no. 4, pp. 619–629, 2001.

[117] L. A. Jerome and V. E. Papaioannou, "DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1," *Nature Genetics*, vol. 27, no. 3, pp. 286–291, 2001.

[118] J. Liao, L. Kochilas, S. Nowotschin et al., "Full spectrum of malformations in velo-cardio-facial syndrome/DiGeorge syndrome mouse models by altering Tbx1 dosage," *Human Molecular Genetics*, vol. 13, no. 15, pp. 1577–1585, 2004.

[119] F. T. Hakim and R. E. Gress, "Reconstitution of the lymphocyte compartment after lymphocyte depletion: a key issue in clinical immunology," *European Journal of Immunology*, vol. 35, no. 11, pp. 3099–3102, 2005.

[120] C. Pignata, M. Fiore, V. Guzzetta et al., "Congenital alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs," *American Journal of Medical Genetics*, vol. 65, no. 2, pp. 167–170, 1996.

[121] L. Auricchio, M. Adriani, J. Frank, R. Busiello, A. Christiano, and C. Pignata, "Nail distrophy associated with a heterozygous mutation of the Nude/SCID human *FOXN1* (*WHN*) gene," *Archives of Dermatology*, vol. 141, no. 5, pp. 647–648, 2005.

[122] A. Amorosi, M. D'armiento, G. Calcagno et al., "FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus," *Clinical Genetics*, vol. 73, no. 4, pp. 380–384, 2008.

[123] I. Vigliano, M. Gorrese, A. Fusco et al., "FOXN1 mutation abrogates prenatal T-cell development in humans," *Journal of Medical Genetics*, vol. 48, no. 6, pp. 413–416, 2011.

[124] E. Kaufmann and W. Knöchel, "Five years on the wings of fork head," *Mechanisms of Development*, vol. 57, no. 1, pp. 3–20, 1996.

[125] T. Schlake, "The nude gene and the skin," *Experimental Dermatology*, vol. 10, no. 5, pp. 293–304, 2001.

[126] M. Schorpp, M. Hoffmann, T. N. Dear, and T. Boehm, "Characterization of mouse and human nude genes," *Immunogenetics*, vol. 46, no. 6, pp. 509–515, 1997.

[127] P. T. Tsai, R. A. Lee, and H. Wu, "BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis," *Blood*, vol. 102, no. 12, pp. 3947–3953, 2003.

[128] G. Balciunaite, M. P. Keller, E. Balciunaite et al., "Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice," *Nature Immunology*, vol. 3, no. 11, pp. 1102–1108, 2002.

[129] V. P. Zediak and A. Bhandoola, "Aging and T cell development: interplay between progenitors and their environment," *Seminars in Immunology*, vol. 17, no. 5, pp. 337–346, 2005.

[130] H. Min, E. Montecino-Rodriguez, and K. Dorshkind, "Reduction in the developmental potential of intrathymic T cell progenitors with age," *Journal of Immunology*, vol. 173, no. 1, pp. 245–250, 2004.

[131] J. Gui, X. Zhu, J. Dohkan, L. Cheng, P. F. Barnes, and D. M. Su, "The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells," *International Immunology*, vol. 19, no. 10, pp. 1201–1211, 2007.

[132] E. Montecino-Rodriquez, H. Min, and K. Dorshkind, "Re-evaluating current models of thymic involution," *Seminars in Immunology*, vol. 17, no. 5, pp. 356–361, 2005.

[133] V. P. Zediak, I. Maillard, and A. Bhandoola, "Multiple prethymic defects underlie age-related loss of T progenitor competence," *Blood*, vol. 110, no. 4, pp. 1161–1167, 2007.

[134] J. Gordon, A. R. Bennett, C. C. Blackburn, and N. R. Manley, "Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch,"

Mechanisms of Development, vol. 103, no. 1-2, pp. 141–143, 2001.

[135] M. Itoi, N. Tsukamoto, and T. Amagai, "Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the postnatal thymus," *International Immunology*, vol. 19, no. 2, pp. 127–132, 2007.

[136] C. C. Blackburn, C. L. Augustine, R. Li et al., "The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 12, pp. 5742–5746, 1996.

[137] C. C. Bleul, T. Corbeaux, A. Reuter, P. Fisch, J. Schulte Monting, and T. Boehm, "Formation of a functional thymus initiated by a postnatal epithelial progenitor cell," *Nature*, vol. 441, no. 7096, pp. 992–996, 2006.

[138] D. Su, S. Navarre, W. Oh, B. G. Condie, and N. R. Manley, "A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation," *Nature Immunology*, vol. 4, no. 11, pp. 1128–1135, 2003.

[139] J. Guo, M. Rahman, L. Cheng, S. Zhang, A. Tvinnereim, and D. M. Su, "Morphogenesis and maintenance of the 3D thymic medulla and prevention of nude skin phenotype require FoxN1 in pre- and post-natal K14 epithelium," *Journal of Molecular Medicine*, vol. 89, no. 3, pp. 263–277, 2011.

[140] S. Shakib, G. E. Desanti, W. E. Jenkinson, S. M. Parnell, E. J. Jenkinson, and G. Anderson, "Checkpoints in the development of thymic cortical epithelial cells," *Journal of Immunology*, vol. 182, no. 1, pp. 130–137, 2009.

[141] L. Cheng, J. Guo, L. Sun et al., "Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy," *Journal of Biological Chemistry*, vol. 285, no. 8, pp. 5836–5847, 2010.

[142] C. C. Blackburn, N. R. Manley, D. B. Palmer, R. L. Boyd, G. Anderson, and M. A. Ritter, "One for all and all for one: thymic epithelial stem cells and regeneration," *Trends in Immunology*, vol. 23, no. 8, pp. 391–395, 2002.

[143] N. R. Manley and C. C. Blackburn, "A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from?" *Current Opinion in Immunology*, vol. 15, no. 2, pp. 225–232, 2003.

[144] M. Itoi, H. Kawamoto, Y. Katsura, and T. Amagai, "Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage," *International Immunology*, vol. 13, no. 9, pp. 1203–1211, 2001.

[145] R. A. Clark, K. Yamanaka, M. Bai, R. Dowgiert, and T. S. Kupper, "Human skin cells support thymus-independent T cell development," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 3239–3249, 2005.

1.3. Molecular evidence for a thymus-independent partial T-cell development in a *FOXN1*$^{-/-}$ athymic human fetus

Genetic alterations of *FOXN1* gene are responsible in humans, mice and rats of the Nude/SCID phenotype, characterized by athymia and severe blockage of T-cell ontogeny. By taking advantage of the opportunity to study tissue samples from a human Nude/SCID fetus, we addressed the issue of defining the role of the gut as alternative site of T-cell ontogeny in a thymus-independent and FOXN1-independent fashion.

Recently, we demonstrated the role of the gut as alternative site of T-cell ontogeny in a thymus-independent and FOXN1-independent fashion. In our study we demonstrate that, in the absence of the thymus, a few rare T lymphocytes are detectable in the cord blood, even though a complete blockage of the $CD4^+$ population was documented (82). In the Nude/SCID fetus, most of the rare $CD3^+$ cells were CD4 and CD8 double negative. A novel population of T cells with similar phenotypic characteristics, $CD3^+B220^{low}CD4^-CD8^-$, exhibiting an impaired proliferative response but producing cytokines and expressing high levels of CD25. In the Nude/SCID, similarly to the control fetus, a $CD3^-CD7^+CD2^+$ population was found, suggesting that the differentiation of this population is FOXN1-independent. In human fetal gut, within the $CD3^-CD7^+$ population a proportion of these cells is able to develop into $CD3^+$ T cells presumably in a thymus-independent manner (88). The identification of $CD7^+$ lymphoid-restricted progenitors in the fetal bone marrow (89) indicates that the CD7 expression and lymphoid commitment occur before the migration to the thymus. Thus far, it has been shown that T-cell precursors and thymus-independent NK-cell precursors start to express the CD7 molecule in fetal liver and bone marrow since the 5th week of gestation (90). Later, $CD7^+$ cells migrate to the thymus where they undergo to

a series of maturational stages eventually developing into fully mature T cells (91). In the absence of the thymus, it is plausible that this population could represent extrathymic originated NK-cell precursor as suggested also by the co-expression of the CD2 molecule. In the epithelium of the small intestine, lymphocytes expressing CD7 and CD2 but not CD3 have already been documented (91). However, the expression of CD7 in the absence of the CD2 marker is generally considered a commitment toward the T-cell lineage. Of note, in the Nude/SCID and the control fetuses, a $CD3^{-}CD7^{+}CD2^{-}$ population was identified, thus supporting the hypothesis that this precursor differentiates toward the T-cell rather than NK-cell lineage. There is also evidence indicating that the majority of the $CD3^{-}CD7^{+}$ population express the CD8αα homodimer and that these cells represent the precursors of the $CD3^{+}$ mature cell population (88). Of note, in mice the expression of the CD8αα homodimer has only been documented on extrathymically derived IELs (87). However, whether cells expressing the CD8 αα homodimer are really locally produced in the intestine or rather they require a functional thymus to be generated is still controversial. In support of a thymus-independent process, in nude mice the presence of $TCR^{+}CD8\alpha\alpha^{+}$T-IELs has been reported and interpreted as a demonstration of a local T-cell differentiation process in the gut. However, these CD8αα T-IELs are much lower in nude mice than in euthymic mice (93). In keeping with this, neonatal thymectomy leads in mice, to a decrease of such cells, similarly to what observed in the Nude mice (94). In our athymic human model we found a negligible number of cells expressing the CD8αα homodimer, thus indicating the requirement of an intrathymic step of differentiation for these cells. Differently, we previously documented in the Nude/SCID human fetus a considerable number of $CD3\varepsilon^{-}CD8\alpha^{+}TCR\gamma\delta^{+}$ cells, which also comprises cells with the CD8αα

heterodimer (82). Under certain circumstances, IELs may express only a partial CD3 complex bearing rare message of the ε chain although the T-cell commitment is established by the presence of pTα transcript (95). Moreover, a number of studies have shown that the TCRs of extrathymically derived T cells are made of either αβ or γδ heterodimers, but the TCR$\gamma\delta^+$ cells are prominent (96). In our study, a definitive demonstration of the local production of T lymphocytes in the intestine in a thymus-independent manner came out from the observation that in the Nude/SCID, $CD3^+$ cells and a few $CD8^+$ cells were detected in the fetal intestine and CD3ε transcript was documented, thus indicating that the process does not require the FOXN1 transcription factor. In particular, $CD3^+$ cells appeared as aggregates in the intestinal crypts. Intestinal T lymphocytes also exhibited a $CD45RA^+$ naive phenotype. As expected, $CD4^+$ cells were absent in either intestine or liver of the Nude/SCID fetus.

The hallmark of the T-cell ontogeny is the TCR gene rearrangement process, characterized by distinctive patterns of gene expression, such as RAG1 and 2 in lymphocytes of both T- and B-cell lineages, which are responsible for the DNA cleavage (97). This process results, in T lymphocytes, in V(D)J recombination**.** Another T lineage specific marker is the pTα expression, in that mature and immature B cells, myeloid cells, NK cells and pluripotent stem cells are pTα negative. This molecule associates with the mature TCR α-chain allowing rearrangement of the α-chain gene (98). The expression of the surrogate TCR chain pTα is upregulated during the DN3 stage of the T-lymphocyte development, along with the expression of the RAG genes (99) pTα-expression is also found in pro-T cells at extra-thymic sites of the T cell development (100). Also in IELs RAG1 and pTα mRNAs are expressed, thus indicating an ongoing TCR gene rearrangement locally in the intestine (101). We found that the

relative expression of RAG1 and RAG2 was approximately 50% of the control and pTα was expressed, as well, accounting for 10% of the control. Although RAG1 and RAG2 enzymes are highly expressed also in pro-B and pre-BII cells, providing the rearrangement of B-cell antigen receptor gene (102), the expression of the pTα gene implies an active process of a thymus-independent T-lymphopoiesis in the intestine in our model. Moreover, we previously documented that in the Nude/SCID cord blood the generation of TCR diversity toke place but was consistently impaired with 2 Vβ families that represented more than 60% of the whole TCR. In this study, we found that the spectratype of intestinal T cells paralleled the cord blood spectratype in that all the families expressed in the CBMC were also expressed in the intestine. This finding indicates that, in the absence of the thymus, the development of intestinal lymphocytes only expressing a limited repertoire occurs as an active process in a thymus-independent fashion. The expression of the αEβ7 integrin CD103 helps direct above originated lymphocytes to migrate into the intestine through cognate interaction with the specific E-cadherin ligand expressed on epithelial cells (103, 104). In this study, the absence of CD103 marker in the intestine further would suggest that the intestinal lymphocytes were not elsewhere originated cells migrated into the intestine.

In humans, alterations of the FOXN1 transcription factor does not interfere with the production of B and NK cells. The normal presence of stem cells, B and NK cells in the Nude/SCID intestine, parallels the relative presence of these cells in the cord blood (105). In contrast, the absence of NK cells in both Nude/SCID and control fetal liver tissues would indicate that NK cell presence in the liver occurs at a later fetal age. Eventually, these findings confirm that at 16 weeks of gestation the development of mature B and NK cells is a thymus-independent process.

To conclude, our results represent the first formal demonstration that an extrathymic lymphopoiesis in the absence of the thymus and in a FOXN1-independent manner occurs in humans, even though it is not sufficient per se for a productive immune response in the peripheral blood.

These data have been published as Article on *PlosOne*, for the manuscript see below.

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Molecular Evidence for a Thymus-Independent Partial T Cell Development in a FOXN1$^{-/-}$ Athymic Human Fetus

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Abstract

The thymus is the primary organ able to support T cell ontogeny, abrogated in FOXN1$^{-/-}$ human athymia. Although evidence indicates that in animal models T lymphocytes may differentiate at extrathymic sites, whether this process is really thymus-independent has still to be clarified. In an athymic FOXN1$^{-/-}$ fetus, in which we previously described a total blockage of CD4$^+$ and partial blockage of CD8$^+$ cell development, we investigated whether intestine could play a role as extrathymic site of T-lymphopoiesis in humans. We document the presence of few extrathymically developed T lymphocytes and the presence in the intestine of CD3$^+$ and CD8$^+$, but not of CD4$^+$ cells, a few of them exhibiting a CD45RA$^+$ naïve phenotype. The expression of CD3εεpTα, RAG1 and RAG2 transcripts in the intestine and TCR gene rearrangement was also documented, thus indicating that in humans the partial T cell ontogeny occurring at extrathymic sites is a thymus- and FOXN1-independent process.

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Introduction

The thymus supports a proper T cell ontogeny due to the presence of specialized epithelial cells, resulting in the export of naive CD45RA$^+$ CD62L$^+$ T cells that follows the recruitment of progenitors from bone marrow [1].

Evidence indicates that T cells may also differentiate at extrathymic sites, as intestine and liver [2–6], where T cell populations may arise from preexisting precursor cells [7,8], even though it still remains to be demonstrated if the process is fully thymus-independent. In favor of a thymic independent differentiation process there is the evidence that a few T cells can be detected into the periphery in nude mice [9–11]. The T cell pool developed outside the thymus exhibits a peculiar phenotype [2] although not univocal in the different species. In fact, in mice, extrathymic T cells often exhibit the CD8αα homodimer, while in rats they may be CD8αβ [12]. In human fetal intestine, T cells are characterized by a higher proportion of TCRγδ$^+$ and CD8αα$^+$ cells [13]. In addition, CD4 and CD8 double negative T cells (CD3$^+$CD4$^-$CD8$^-$) isolated from the intestine are generally considered of extrathymic origin [13]. In the epithelium of the small intestine, lymphocytes may also express CD7 and CD2 in the absence of CD3 (CD2$^+$CD3$^-$CD7$^+$). In humans, the expression of RAG in the gut indicates that at this site a gene rearrangement process may take place, suggesting an active lymphopoiesis [14].

FOXN1 is a developmentally regulated transcription factor, selectively expressed in epithelial cells of the skin and thymus, where it plays a necessary role for T lymphopoiesis [15–17] by inducing a proper epithelial cell differentiation and endothelial cell/thymic mesenchyme communication network [18]. FOXN1 mutations lead to athymia [19,20] and result, in humans, in a SCID phenotype, referred as the human equivalent of the mice Nude/SCID syndrome [21–24]. During early prenatal life in humans, homozygous FOXN1 mutation leads to a complete blockage of the CD4$^+$ T cell maturation, while a few CD8α$^+$TCRγδ$^+$ cells, not expressing CD3ε molecule and not able to respond to a mitogenic stimulation, are found [25], thus suggesting an extrathymic site of lymphopoiesis for these cells.

Here we studied the role of the intestine and liver as extrathymic sites of thymus-independent and FOXN1-independent T lymphopoiesis in a FOXN1$^{-/-}$ athymic human fetus. We found the presence of a few T cells with a peculiar phenotype, indicative of the thymus-independent lymphopoiesis.

Results and Discussion

Detection of extrathymically derived T lymphocytes in the cord blood of FOXN1$^{-/-}$ fetus

The fetus analyzed in the present study was identified during a genetic counseling offered to heterozygous couples at risk for Nude/SCID disease, originated in the same geographic area where the first patients were identified [26]. The specific defect

(R255X mutation in the FOXN1 gene) was searched on chorionic villi by direct sequencing.

In the absence of the thymus, few lymphocytes in CB co-express $CD7^+CD2^+$ (12% of $CD3^-$ gated lymphocytes) in the $FOXN1^{-/-}$ fetus, as compared to the control (17.2%) (Figure 1A). This population also comprises NK cells.

Extrathymic derived intraepithelial lymphocytes (IELs) are difficult to be univocally characterized, in that in mice they preferentially bear TCRγδ and express the CD8αα [11,27,28], while in rats they express the CD8αβ heterodimer [12]. We previously described that in the $FOXN1^{-/-}$ CBMCs, most of the $CD8^+$ cells were $CD3^-$ [25], thus we looked at the $CD8\alpha\alpha^+$ cells on $CD3^-$ gated lymphocytes. These cells were 1.3% in the $FOXN1^{-/-}$ CBMCs and much more represented in the control (21.7%) (Figure 1A). Our data are in favor of a thymic dependence of such cells. In nude mice, a number of $TCR^+CD8\alpha\alpha^+$ T IELs, lower than what found in euthymic mice, has also been reported [11].

The absence of CD4 molecule, would argue against the possibility that the $CD3^-CD8\alpha^+$ cells were dendritic cells (DC) [29]. Moreover, since the $CD3^-CD8\alpha^+$ cells were analyzed setting the gate on lymphoid cells, this would rule out the possibility that they were DCs of myeloid origin. In addition, the $CD3^-CD8\alpha^+$ cells are unlikely to be NK cells, in that they should express the CD8 with a dim intensity instead of a CD8 with a bright intensity, as in T cells, similarly to what found in Nude/SCID fetus.

In the $FOXN1^{-/-}$ fetus, most of the rare $CD3^+$ cells were CD4 and CD8 double negative (56.2% of $CD3^+$ gated lymphocytes) as compared to the control (4.2% of $CD3^+$ gated lymphocytes) (Figure 1A). A novel population of T cells with a similar phenotype, $CD3^+B220^{low}CD4^-CD8^-$, has also been identified in a *nu/nu* mouse, suggesting an extrathymic origin [30].

We previously documented in the $FOXN1^{-/-}$ human fetus a considerable number of $CD3\varepsilon^-CD8\alpha^+TCR\gamma\delta^+$ cells, which also comprises cells with the CD8αβ heterodimer [25]. IELs may express only a partial CD3 complex bearing rare message of the ε

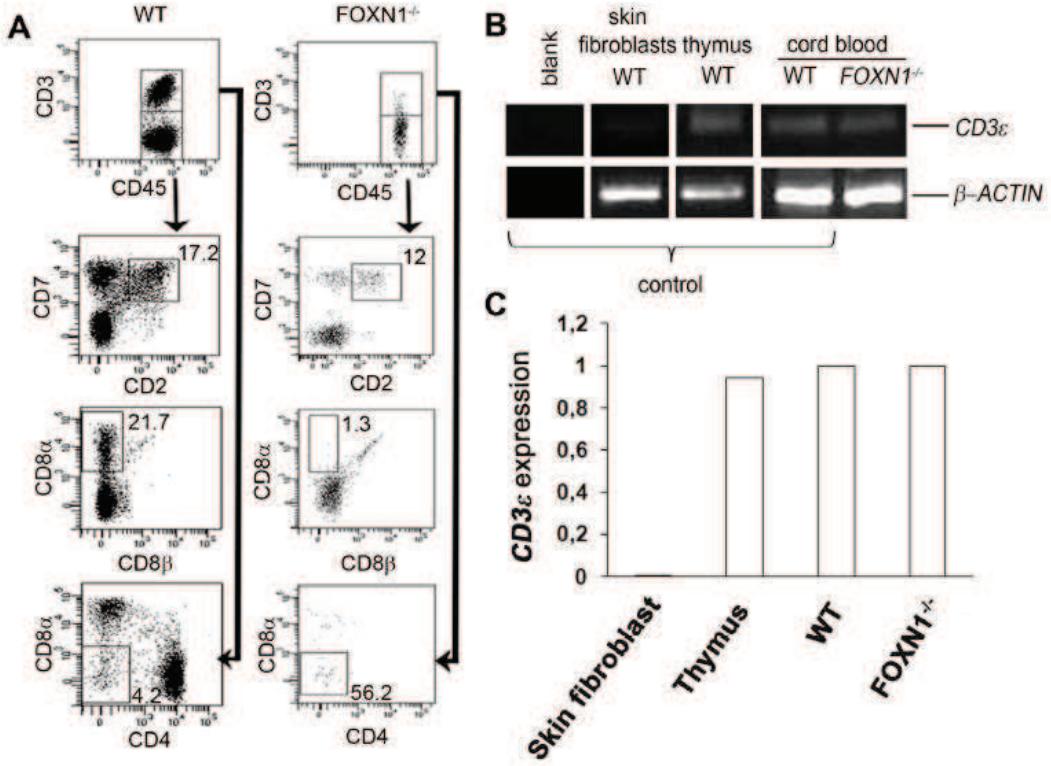

Figure 1. Detection of extrathymically derived T lymphocytes in the cord blood of $FOXN1^{-/-}$ human fetus. (A) Flow cytometry analysis of CBMCs from WT (left dot plots) or $FOXN1^{-/-}$ (right dot plots) fetuses (16 weeks of gestation). CD7 and CD2 together with the CD8α and CD8β expression patterns for the gated $CD45^+CD3^-$ cells are shown. CD8α and CD4 expression is shown for the gated $CD45^+CD3^+$ cells. Numbers indicate the frequency of the cells within the gate. Experiment was repeated two times. Data were obtained by gating first on viable cells and later on $CD45^+$ cells. (B) RT-PCR analysis of CD3ε expression in CBMCs. The expression of CD3ε transcript in human skin fibroblasts (negative control), human thymus (positive control), CBMCs from WT or $FOXN1^{-/-}$ fetuses is shown. Blanck, no cDNA. β-actin was used as loading control. Representative results from three independent experiments are shown. (C) Quantitative real-time PCR showing the expression of mRNAs encoding CD3ε (relative to β-actin) in skin fibroblasts (negative control), thymus (positive control) and CBMCs from WT or $FOXN1^{-/-}$ fetuses (16 weeks of gestation). Representative results from two independent experiments are shown.
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chain although the T cell commitment is established by the presence of pTα transcript [31,32]. Thus, we evaluated the median fluorescence intensity (MFI) of CD3 signal, which was much lower in the FOXN1$^{-/-}$ fetus than in the control (213 versus 1275 MFI, respectively), in keeping with the already reported dim signal in the same FOXN1$^{-/-}$ fetus [25]. The presence in the FOXN1$^{-/-}$ fetus of CD3$^+$ cells, was however confirmed by the presence of the CD3ε transcript (Figure 1B). It should be noted that mRNA expression is almost equivalent in both FOXN1$^{-/-}$ cells and wild type (Figure 1C). Thus, we cannot exclude that CD3$^-$ cells are really CD3ε^{low} cells.

Extrathymic sites of B-, NK- and T-lymphopoiesis in FOXN1$^{-/-}$ SCID human fetus

Since in humans, intestine and liver are considered the main organs for extrathymic lymphopoiesis [2], we characterized the lymphocytes in these tissues. As expected on the T^{low}B$^+$NK$^+$ phenotype of the human Nude/SCID [21], CD34$^+$ cells and B cells (CD20) were normal in tissue sections (Figure 2A). The CD56 marker for NK cells revealed the presence of few and spread positive cells in the intestine sections but not in the liver (Figure 2A). Eventually, these findings confirm that, at 16 weeks of gestation, the development of mature B and NK cells is a thymus-independent process. Moreover, also the morphology of intestine and liver sections, evaluated through H&E staining, was normal (Figure 2B).

Within FOXN1$^{-/-}$ intestine tissue, CD3$^+$ cells were spread in the mucosa with a trend to aggregate in the crypts while in the control they formed clear aggregates (Figure 2B). In the liver of both FOXN1$^{-/-}$ and control, CD3$^+$ cells were present but spread (Figure 2B). Accordingly to what found in CB, CD4$^+$ cells were absent in either intestine and liver of the FOXN1$^{-/-}$ fetus, differently from the control (Figure 2B). A few CD8$^+$ cells were detected in the FOXN1$^{-/-}$ intestine similarly to the control (Figure 2B). Quantification in 5 random fields of the positive cells, stained as in Figure 2B, confirmed the absence of CD4$^+$ cells and the presence of few CD8$^+$ cells in both tissues of the FOXN1$^{-/-}$ fetus (19.6±3 in FOXN1$^{-/-}$ intestine versus 27.4±2 in WT intestine, $p \leq 0.05$; 9.8±2 in FOXN1$^{-/-}$ liver versus 14.6±4 in WT liver). No double positive (CD4$^+$CD8$^+$) thymocytes were found by confocal microscopy in the FOXN1$^{-/-}$ intestine (Figure 2C). When CD7$^+$ cells were also stained for CD3, a few CD3$^-$CD7$^+$ cells were detected in the intestine, even though the majority of them co-expressed both molecules (Figure 2D). In the FOXN1$^{-/-}$ fetus we demonstrated the presence of the CD3ε transcript through RT-PCR amplification of intestinal mRNA (Figure 2E). The quantitative PCR analysis revealed that the amount of this molecule in the intestine of FOXN1$^{-/-}$ fetus was even higher than in the control (Figure 2F). Taken together these data suggest that a local production of T lymphocytes takes place in the intestine and liver in a thymus- and FOXN1-independent manner, even though we cannot completely exclude an early contribution of a thymus primordium to the production of T cells.

Cells with naive phenotype can develop in the FOXN1$^{-/-}$ human athymic fetus

The CD45RA molecule and the L-Selectin CD62L are considered markers of Recent Thymic Emigrants (RTE), thus being the hallmark of naive lymphocytes. In FOXN1$^{-/-}$ CBMCs, 3.3% of CD45$^+$ gated cells co-expressed CD3, with a dim intensity, and the CD45RA, differently from the control, in whom this population was 35.9%, almost all expressing CD3 with bright intensity (Figure 3A). On CD45$^+$ gated cells, only a negligible number co-expressed the CD3 and CD62L markers (0.8%), as compared to the 10.8% of the control (Figure 3B). In FOXN1$^{-/-}$ CBMCs, 22.8% of CD3$^+$ cells co-expressed both CD62L and CD45RA, similarly to the control (Figure 3C). The analysis of CD27 associated with CD45RA, as a further marker of a naive cell phenotype [33,34], revealed the presence of CD27$^+$CD45RA$^+$ cells (13.4% of CD3$^+$ gated cells) in FOXN1$^{-/-}$ CBMCs (Figure 3D). The immunofluorescence co-staining of CD45RA and CD3 molecules revealed in the intestine of the FOXN1$^{-/-}$ fetus the presence of cells co-expressing both molecules (Figure 3E). This finding indicates that intestinal T lymphocytes also exhibit a naive phenotype.

Identification of intestinal de novo lymphopoiesis in the FOXN1$^{-/-}$ athymic fetus

TCR gene rearrangement occurs at the T cell precursor stage and results in a functional antigen receptor. The process requires RAG1 and RAG2 recombination activity, which results in pTα production. The fate of pTα-expressing progenitors was found to include all αβ and most γδ T cells but to exclude B, NK, and thymic dendritic cells [32]. The expression of the surrogate TCR chain pTα is upregulated during the DN3 stage of the T lymphocyte development, along with the expression of the RAG genes. pTα expression is also found in pro-T cells at extrathymic sites of the T cell development in Nude mice [35]. Also in IELs RAG1 and pTα mRNAs are expressed, thus indicating an ongoing TCR gene rearrangement locally in the intestine [14,36]. In the FOXN1$^{-/-}$ fetus, the relative expression of RAG1 and RAG2 mRNA was 47.5 and 68.4% of the control, respectively (Figure 4A), whereas pTα even though to a lesser extent, is detectable, accounting for 20.0% of the control (Figure 4A), thus suggesting that in the absence of the thymus the rearrangement occurs in the FOXN1$^{-/-}$ intestine, but the process is only limited to few T cells. These results, along with the CD3ε expression, suggest the presence of a de novo intestinal production of T cells. While RAG1 and RAG2 enzymes are highly expressed also in pro-B and pre-BII cells, the expression of the pTα is in favor of a thymus-independent T-lymphopoiesis.

In this study, we found that the TCR repertoire of FOXN1$^{-/-}$ intestinal lymphocytes paralleled the CBMCs spectratype, which was consistently impaired [25]. A statistically significant quantitative correlation was found in the contribution of all Vβ families to the TCR repertoire ($r = 0.78$; $p < 0.001$), but of Vβ14, which represented the only family, with an intestinal expression higher than 10% of the total mRNA, not being expressed at all in CBMCs (Figure 4B). The lower expression of the pTα in the FOXN1$^{-/-}$ intestine, as compared to the control, along with the altered TCR spectratyping, suggest that only a partial T cell ontogeny occurs in the intestine, limited to very few families and resulting in a limited repertoire, generated in a thymus-independent fashion.

T cells localized in the epithelium of skin, gut, lung and allograft tissues are characterized by the expression of the αEβ7 integrin CD103, which is involved in directing previously stimulated lymphocytes, above originated, to epithelial cells [37]. No CD3$^+$CD103$^+$ cells were detected in both FOXN1$^{-/-}$ fetus CB (0.3% of CD45$^+$ gated cells) (Figure 5A), and at the immunohistochemical evaluation of FOXN1$^{-/-}$ intestine tissue (not shown), suggesting that intestinal T cells in the FOXN1$^{-/-}$ fetus were locally produced. Moreover, in the FOXN1$^{-/-}$ CBMCs, most of T cells didn't express the CD45RO activation marker (Figure 5B), indicating that they had not previously encountered any antigen.

In conclusion, although there is still the possibility that a thymus anlage contributes to produce the few T cells observed in the

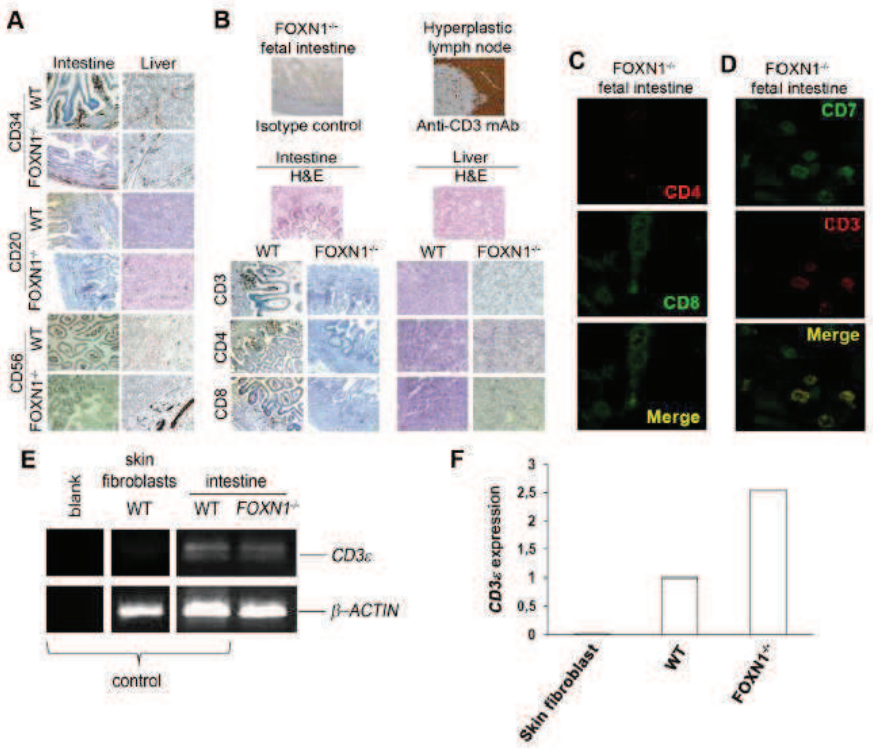

Figure 2. Identification of lymphocytes at extrathymic sites of differentiation in a Nude/SCID human fetus. (A, B) Immunohistochemical detection of lymphocytes at extrathymic sites of differentiation in FOXN1$^{-/-}$ human fetus (16 weeks of gestation). (A) Stem cells, B cells and NK cells were detected by immunohistochemical stain for CD34 (brown), CD20 (brown) and CD56 (brown) in intestinal and liver sections obtained from a FOXN1$^{-/-}$ human fetus (16 weeks of gestation) or an aged-matched control fetus. In CD34 and CD20 stained intestine sections from WT and FOXN1$^{-/-}$ fetuses original magnification was 100x. (B) As negative control, intestinal sections from FOXN1$^{-/-}$ fetus were counterstained with hematoxylin and with the isotype control (primary antibody omitted) by DAB. As positive control, hyperplastic lymph node sections were stained for CD3 (brown) by DAB. Intestinal or liver sections of FOXN1$^{-/-}$ human fetus were counterstained with hematoxylin and eosin (H&E). Immunohistochemical analysis of intestinal or liver sections of a FOXN1$^{-/-}$ human fetus and an aged-matched control fetus using anti-CD3 staining to mark T cells, anti-CD4 staining to mark T helper cells and anti-CD8 staining to mark cytotoxic T cells. DAB, 200x. Representative results from two independent experiments with two distinct samples are shown. (C, D) Confocal microscopy of FOXN1$^{-/-}$ intestinal sections. (C) Labeling with anti-human CD4 (red) and anti-human CD8 (green). (D) Labeling with anti-human CD7 (green) and anti-human CD3 (red). Representative results from three independent experiments are shown. (E) RT-PCR analysis of CD3ε intestinal expression. CD3ε transcript expression in human skin fibroblasts (negative control), intestinal lymphocytes from WT or FOXN1$^{-/-}$ fetuses is shown. Blanck, no cDNA. β-actin was used as loading control. Representative results from three independent experiments are shown. (F) Quantitative real-time PCR showing the expression of mRNAs encoding CD3ε (relative to β-actin) in skin fibroblasts (negative control), thymus (positive control) and intestinal tissue of control and FOXN1$^{-/-}$ fetuses (16 weeks of gestation). Representative results from two independent experiments are shown.
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FOXN1$^{-/-}$ patients, our results support the hypothesis that T cells do mature at extrathymic sites with an alternative lymphopoietic process, involving the same molecules implicated in intrathymic development, as pTα and RAGs. This process in humans is thymus- and FOXN1-independent. In summary, we document that a few T lymphocytes with a peculiar phenotype may develop in a thymus- and FOXN1-independent manner. We also report on the presence of intestinal CD3$^+$ and CD8$^+$, but not CD4$^+$ cells, a few of them showing a naive phenotype. The expression of CD3εε pTα, RAG1 and RAG2 transcripts in the intestine and TCR gene rearrangement, although abnormal, indicates that in humans a partial T cell ontogeny occurs at extrathymic sites in the Nude/SCID phenotype in a FOXN1-independent manner.

Materials and Methods

Fetus samples

Cord blood (CB) from the FOXN1$^{-/-}$ fetus was obtained by cordocentesis at 16 weeks of gestation. Experiments using CB or fetal tissue samples were approved by the Institutional Ethical Committee for Biomedical Activities "Carlo Romano" at the "Federico II" University of Naples. Age-matched CB cells from the CEINGE bank were used as control. Fetus parents provided written informed consent.

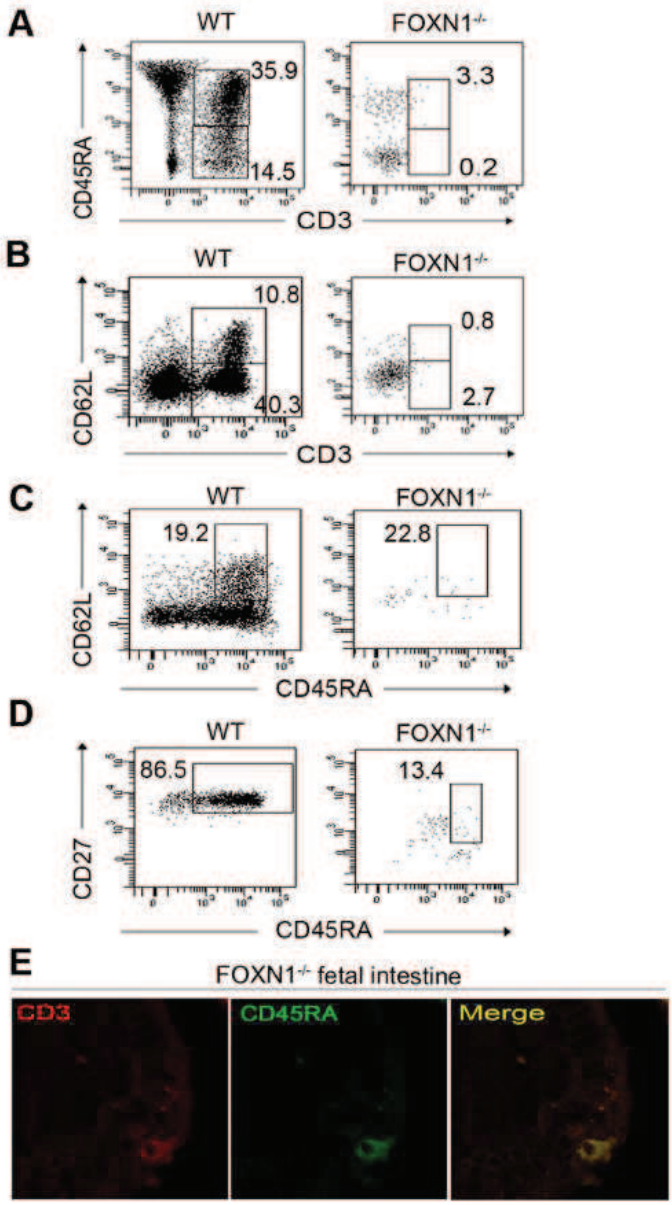

Figure 3. Lymphocytes with naive phenotype in cord blood and intestine. Flow cytometry of CBMCs from normal and FOXN1$^{-/-}$ fetuses matched for gestational age (16 weeks of gestation). Dot plots show the expression pattern of the naïve cell markers. (A) Frequencies of CBMCs expressing both CD45RA and CD3 markers. (B) Frequencies of CBMCs expressing both CD62L and CD3 markers. (C) Frequencies of CBMCs coexpressing CD45RA and CD62L markers. (D) Frequencies of CBMCs coexpressing CD45RA and CD27 markers. Experiment in (A), (B), (C) and (D) was repeated two times. Data were obtained by gating first on viable cells and later on CD45$^+$ cells (A and B) or finally also on CD3$^+$ (C and D). (E) Confocal microscopy of fetal intestinal sections labeled with anti-human CD3 (red) and anti-human CD45RA (green). Representative results from three independent experiments with two samples are shown.
doi:10.1371/journal.pone.0081786.g003

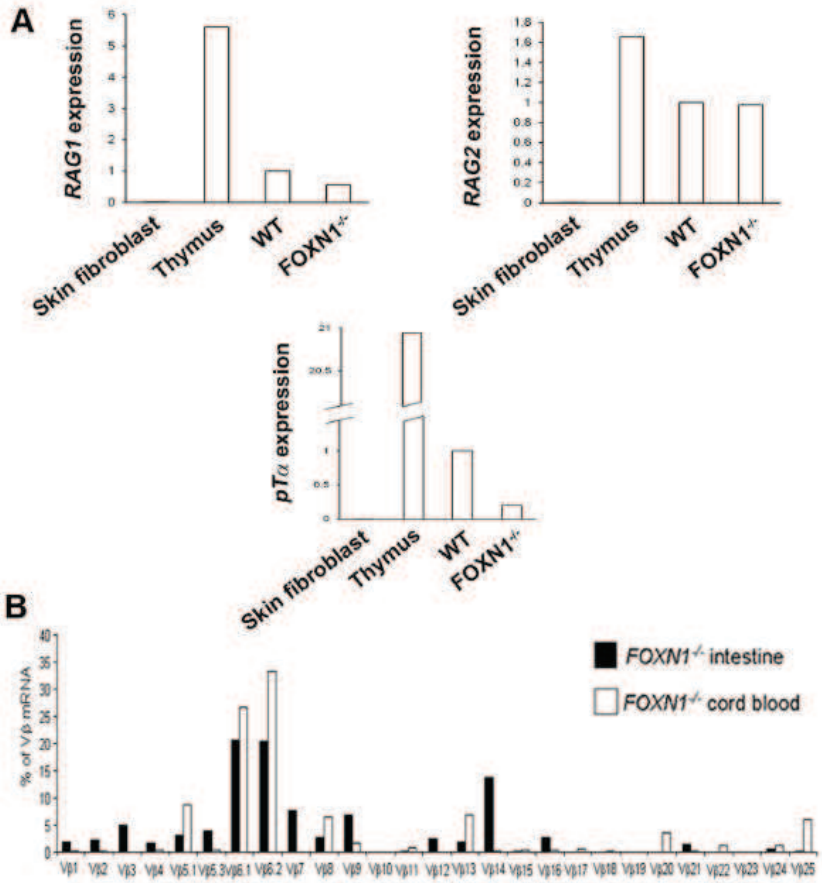

Figure 4. Local production of T cells in the FOXN1$^{-/-}$ human intestine. (A) Quantitative real-time PCR showing the expression of mRNAs encoding RAG1, RAG2 and pTα (relative to β-actin) in skin fibroblasts (negative control), thymus (positive control) and intestinal tissue of control and FOXN1$^{-/-}$ fetuses (16 weeks of gestation). (B) Comparison of TCR Vβ-region usage between intestinal lymphocytes (black bars) and CBMCs (white bars) from the FOXN1$^{-/-}$ fetus. Experiments were repeated three times in (A) and two times in (B).
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Cell-surface staining and flow cytometry

In flow cytometry, FITC-, phycoerythrin (PE)-, allophyocyanin-cyanine 7 (APC-Cy7)-, peridin chlorophyll protein (PerCP)- or PE-Cy7-coupled Abs were used on CB toward CD45 (2D1), CD7 (M-T701), CD2 (RPA-2.10), CD3 (UCHT1), CD8α (SK-1), CD8β (2ST8.5H7), CD4 (L200), CD62L (SK11), CD45RA (HI100), CD27 (L128), CD45RO (UCHL-1), CD103 (Ber-ACT8) from BD Pharmingen, San Diego, CA or Beckman Coulter, Brea, CA. FACSCanto II flow cytometer and FACSDiva software (BD Bioscience, San Jose, CA) were used. For each sample, negative controls were stained with irrelevant Abs conjugated with the same fluorochrome [38]. The "fluorescence-minus-one" (FMO) controls have also been used to define precisely the cells that have fluorescence above background levels. Briefly, the samples have been stained with all of the reagents except one [38].

Histology

Intestine and liver tissue samples from a 16 weeks FOXN1$^{-/-}$ fetus or control were embedded in OCT compound and snapfrozen in liquid nitrogen or paraffin-embedded. The blocks were cut into serial 5-µm sections and mounted onto microscope slides for H&E staining and immunohistochemistry analysis. Immunodetections were performed by means of a Ventana automat (Ventana Medical Systems, Illkirch, France).

Immunohistochemistry

Tissue sections staining was performed on Benchmark XT platform (Ventana Medical Systems) with pre-diluted CD34, CD20, CD56, CD3, CD4 (Ventana-Confirm), CD8 (Cell Marque), 1:40 CD103 (Beckman Coulter, Brea, CA), 1:50 CD45RA (Dako, Denmark) and 1:25 CD62L Abs (Abcam, Cambridge, UK). Heat antigen retrieval was performed in buffer (CC1, Ventana) following the manufacturer instructions. The slides were incubated with primary Abs at 37° for 32 min (CD34,

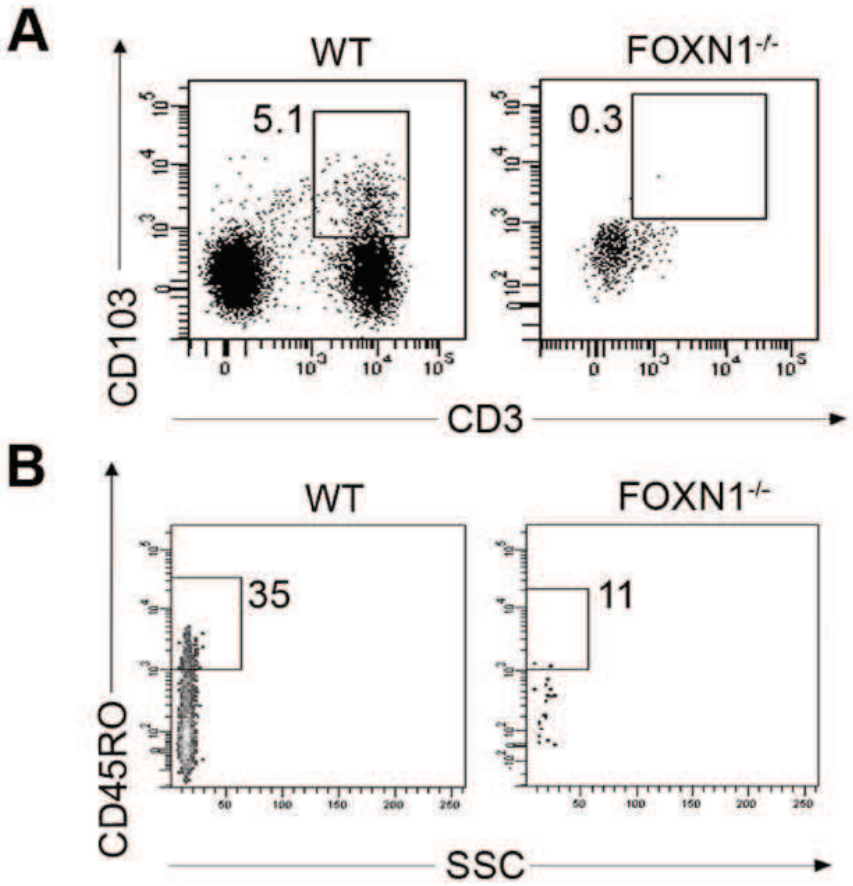

Figure 5. Expression on T lymphocytes from FOXN1$^{-/-}$ and control fetuses of the integrin CD103 and the CD45RO activation marker. (A, B) Flow cytometry of CBMCs from normal and FOXN1$^{-/-}$ fetuses matched for gestational age (16 weeks of gestations). Dot plots show the frequency of CD103^{+}CD3^{+} on CD45^{+} gated CBMCs (A) and CD45RO^{+} on CD3^{+} gated CBMCs (B).
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CD20, CD56, CD3, CD4 and CD8) or for 60 min (CD45RA, CD103, CD62L). Primary Ab was omitted for negative control. Nuclei were counterstained with hematoxylin. The reaction was detected by the ultraView Universal DAB Detection Kit, which utilizes a cocktail of enzyme labeled secondary Abs that locates the bound primary Ab. The complex is then visualized with hydrogen peroxide substrate and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a dark brown precipitate readily detected by light microscopy. Images were acquired by a microscope (DM 2500; Leica, Germany) at magnification 200 x or 100 x.

Confocal microscopy

Tissue samples were blocked with normal goat serum before staining and then treated with 1:50 of PerCP-labeled CD3 (BD Pharmingen, San Diego, CA) and 1:100 of FITC-labeled CD45RA Abs (BD Pharmingen, San Diego, CA) or 1:50 of PE-labeled CD4 (Beckman Coulter, Brea, CA) and 1:50 FITC-labeled CD8 (Beckman Coulter, Brea, CA) or 1:50 APC-labeled CD3 (Beckman Coulter, Brea, CA) and FITC-labeled CD7 (Beckman Coulter, Brea, CA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

RNA and RT-PCR

Total RNA was isolated from normal human skin fibroblasts, normal human thymus, CB mononuclear cells (CBMCs) or intestinal frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and the Phase-lock gel columns (Eppendorf) by standard procedures. RNA was reverse transcribed by SuperScript III reverse transcription (Invitrogen, Carlsbad, CA). RT-PCR was performed using Taq polymerase (Roche, Germany). The following primers were used to amplify CD3ε: (forward) 5′-GATGCAGTCGGGCACTCACT-3′ and (reverse) 5′-TTGGGGGCAAGATGGTAATG-3′); or β-actin: (forward) 5′-GACAGGATGCAGAAGGAGAT-3′ and (reverse) 5′-TTGCTGATCCACATCTGCTG-3′. To avoid amplification of genomic DNA, the reverse primer for CD3ε was located on the 3-4 exons junction.

Evaluation of TCR β-chain variable region (Vβ) spectratyping

TCR CDR3β sequencing of total mRNA isolated from intestine of the FOXN1$^{-/-}$ or control fetuses was performed after TCR β-chain amplification with a common reverse primer (CB3 primer) and 27 different forward primers (TCR Vβ gene family primers). PCR products were run on a CEQ 8000 automatic capillary sequencer (Beckman Coulter, Brea, CA) and fractionated on the size of the CDR3 region. Results were analyzed using CEQ 8000 software (Beckman Coulter, Brea, CA), which also gives the percentage contribution of a single family to the total TCR repertoire.

Quantitative real-time PCR

Real-time PCR was performed using the SYBR green detection reagent and analyzed with the Light Cycler480 system (Roche, Germany). Genes were normalized to β-actin as housekeeping gene and the relative messenger RNA expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method [39]. The following primers were used to amplify β-actin: (forward) 5′-GACAGGATGCAGAAGGAGAT-3′ and (reverse) 5′-TTGCTGATCCACATCTGCTG-3′; or CD3ε: (forward) 5′-GATGCAGTCGGGCACTCACT-3′ and (reverse) 5′-TTGGGGGCAAGATGGTAATG-3′; or RAG1: (forward) 5′-CATCAAGCCAACCTTCGACAT-3′ and (reverse) 5′-CAGGACCATGGACTGGATATCTC-3′; or RAG2: (forward) 5′-CCTGAAGCCAGATATGGTC-3′ and (reverse) 5′-GTGCAATTCACAGCTGGGCT-3′; or pTα: (forward) 5′-CATCCTGGGAGCCTTTGGT-3′ and (reverse) 5′-CCGGTGTCCCCCTGAGAG-3′. The pTα reverse primer was located on the 3-4 exons junction to avoid DNA contamination.

Statistical analysis

GraphPad Prism software was used for data analysis. The *t*-student test was used to analyze the statistical significance of differences. The minimum acceptable level of significance was $p \leq 0.05$.

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Author Contributions

Conceived and designed the experiments: AF CP. Performed the experiments: AF LuP MG GB LV GS. Analyzed the data: AF CP RD RR LoP LDV. Contributed reagents/materials/analysis tools: CP LuP GB LDV LV MVB VG. Wrote the paper: AF CP.

References

1. Saran N, Pommerencke J, Witzlau K, Regelin M, Krueger A (2012) Extrathymic physiological T lineage progenitor activity is exclusively confined to cells expressing either CD127, CD90, or high levels of CD117. Plos One 7: e30864.
2. Torfadottir H, Freysdottir J, Skaftadottir I, Haraldsson A, Sigfusson G, et al. (2006) Evidence for extrathymic T cell maturation after thymectomy in infancy. Clin Exp Immunol 145: 407–412.
3. Nonaka S, Naito T, Chen H, Yamamoto M, Moro K, et al. (2005) Intestinal γδ T cells develop in mice lacking thymus, all lymphonodes, Peyer's patches, and isolated lymphoid follicles. J Immunol 174: 1906–1912.
4. Iiai T, Watanabe H, Seki S, Sugiura K, Hirokawa K, et al. (1992) Ontogeny and development of extrathymic T cells in mouse liver. Immunology 77: 556–563.
5. Sato K, Ohtsuka K, Hasegawa K, Yamagiwa S, Watanabe H, et al. (1995) Evidence for extrathymic generation of intermediate T cell receptor cells in the liver revealed in thymectomized, irradiated mice subjected to bone marrow transplantation. J Exp Med 182: 759–767.
6. Blais M, Louis I, Corneau S, Gerard G, Terra R, et al. (2003) Extrathymic T-lymphocyte development. Exp Hematol 31: 349–354.
7. Sugahara S, Shimizu T, Yoshida Y, Aiba T, Yamagiwa S, et al. (1999) Extrathymic derivation of gut lymphocytes in parabiotic mice. Immunology 96: 57–65.
8. Parker GA, Picut CA (2005) Liver Immunobiology. Toxicol Pathol 33: 52–62.
9. Bandeira A, Itohara S, Bonneville M, Burlen-Defranoux O, Mota-Santos T, et al. (1991) Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor gd. Proc Natal Acad Sci USA 88: 43–47.
10. Kennedy JD, Pierce CW, Lake JP (1992) Extrathymic T cell maturation. Phenotypic analysis of T cell subsets in nude mice as a function of age. J Immunol 148: 1620–1629.
11. Rocha B (2007) The extrathymic T-cell differentiation in the murine gut. Immunol Rev 215: 166–177.
12. Torres-Nagel N, Kraus E, Brown MH, Tiefenthaler G, Mitnacht R, et al. (1992) Differential thymus dependence of rat CD8 isoform expression. Eur J Immunol 22: 2841–2848.
13. Gunther U, Holloway JA, Gordon JG, Knight A, Chance V, et al. (2005) Phenotypic characterization of CD3-7+ cells in developing human intestine and an analysis of their ability to differentiate into T cells. J Immunol 174: 5414–5422.
14. Lundqvist C, Baranov V, Hammarström S, Athlin L, Hammarström ML. (1995) Intra-epithelial lymphocytes. Evidence for regional specialization and extra-thymic T cell maturation in the human gut epithelium. Int Immunol 7: 1473–1487.
15. Coffer PJ, Burgering BMT (2004) Forkhead-box transcription factors and their role in the immune system. Nat Rev Immunol 4: 889–899.
16. Romano R, Palamaro L, Fusco A, Iannace L, Maio S, et al. (2012) From murine to human Nude/SCID: the Thymus, T-cell development and the missing link. Clin Dev Immunol 2012: 467101.
17. Palamaro L, Guarino V, Scalia G, Antonini D, De Falco L, et al. (2013) Human skin-derived keratinocytes and fibroblasts co-culture on 3D poly encaprolactone scaffold support *in vitro* HSCs differentiation into T-lineage committed cells Int Immunol In press.
18. Bryson JL, Griffith AV, Hughes Iii B, Saito F, Takahama Y, et al. (2013) Cell-autonomous defects in thymic epithelial cells disrupt endothelial-perivascular cell interactions in the mouse thymus. Plos One 8: e65196.
19. Schorpp M, Hoffmann M, Dear TN, Boehm T (1997) Characterization of mouse and human nude genes. Immunogenetics 46: 509–515.
20. Flanagan SP (1966) 'Nude', a new hairless gene with pleiotropic effects in the mouse. Genet Res 8: 295–309.
21. Pignata C, Fiore M, Guzzetta V, Castaldo A, Sebastio G, et al. (1996) Congenital alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. Am J Med Genet 65: 167–170.
22. Pignata C, Gaetaniello L, Masci AM, Frank J, Christiano A, et al. (2001) Human equivalent of the mouse nude/SCID phenotype: Long-term evaluation of immunological reconstitution after bone marrow transplantation. Blood 97: 880–885.
23. Albuquerque A, Marques JG, Silva SL, Ligeiro D, Devline BH, et al. (2012) Human FOXN1-deficiency is associated with αβ double-negative and FoxP3+ T-cell expansions that are distinctly modulated upon thymic transplantation. Plos One 7: e37042.
24. Pignata C, Fusco A, Amorosi S (2009) Human clinical phenotype associated with FOXN1 mutations. Adv Exp Med Biol 665: 195–206.
25. Vigliano I, Gorrese M, Fusco A, Vitiello L, Amorosi S, et al. (2011) FOXN1 mutation abrogates prenatal T-cell development in humans. J Med Genet 48: 413–416.
26. Adriani M, Martinez-Mir A, Fusco F, Busiello R, Frank J, et al. (2004) Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. Ann Hum Genet 68: 265–268.
27. Ishikawa H, Naito T, Iwanaga T, Takahashi-Iwanaga H, Suematsu M, et al. (2007) Curriculum vitae of intestinal intraepithelial T cells: their developmental and behavioral characteristics. Immunol Rev 215: 154–165.
28. Guy-Grand D, Azogui O, Celli S, Darche S, Nussenzweig MC, et al. (2003) Extrathymic T Cell Lymphopoiesis: Ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. J Exp Med 197: 333–341.
29. Martin P, del Hoyo GM, Anjure F, Ruiz SR, Arias CF, et al. (2000) Concept of lymphoid versus myeloid dendritic cell lineages revised: both CD8α^{-} and CD8α^{+} dendritic cells are generated from CD4^{low} lymphoid-committed precursors. Blood 96: 2511–2519.
30. Johansson M, Lycke N (2003) A unique population of extrathymically derived (alpha)-(beta) TCR+CD4-CD8- T cells with regulatory functions dominates the mouse female genital tract. J Immunol 170: 1659–1666.
31. Oida T, Suzuki K, Nanno M, Kanamori Y, Saito H, et al. (2000) Role of Gut Cryptopatches in Early Extrathymic Maturation of Intestinal Intraepithelial T Cells. J Immunol 164: 3616–3626.

32. Luche H, Nageswara Rao T, Kumar S, Tasdogan A, Beckel F, et al. (2013) In vivo fate mapping identifies pre-TCRa expression as an intra- and extrathymic, but not prethymic, marker of T lymphopoiesis. J Exp Med 210: 699–714.
33. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401: 708–712.
34. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, et al. (1997) Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med 186: 1407–1418.
35. Bruno L, Rocha B, von Boehmer H, Rodewald H-R (1995) Intra- and extra-thymic expression of the pre-T cell receptor a gene. Eur J Immunol 25: 1877–1882.
36. Bas A, Hammarstrom SG, Hammarstrom ML-KC (2003) Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. J Immunol 171: 3359–3371.
37. Uss E, Rowshani AT, Hooibrink B, Lardy NM, van Lier RAW, et al. (2006) CD103 is a marker for alloantigen-induced regulatory CD8+ T cells. J Immunol 177: 2775–2783.
38. Perfetto SP, Chattopadhyay PK, Roederer M (2004) Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol 4: 648–655.
39. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402–408.

1.4. A "thymic organoid" leads to differentiation toward T-lineage committed cells *in vitro*

The thymus capability to lead differentiation of hemopoietic stem cells (HSCs) into T lymphocytes is mediated by specialized TECs. The three-dimensional organization of thymic stromal cells maximizes the interaction of developing thymocytes with TECs, allowing intercellular cross-talk required to development of both T cells and TECs (106).

A remarkable number of similarities are shared between the epithelial and stromal cells of the thymus and keratinocytes and fibroblasts of the skin. Both thymic and skin epithelial cells selectively express the FOXN1 transcription factor, which plays a critical role in differentiation and survival of these specialized cells (107, 108). Hassall corpuscles, a product of medullary thymic epithelial cells, contain keratins identical to those in the stratum corneum of skin. In addition, keratinocytes express many of same keratins expressed in TECs (109). Moreover, the Notch pathway is shared between thymic stroma and skin elements, since it plays an important role in regulating epidermal differentiation, in early stages of thymocyte maturation and T-cell lineage commitment (110). By contrast, a profound difference between thymus and skin is the architecture of each organ, in that the skin epithelial cells are organized in a two-dimensionally (2D) oriented fashion differently from epithelial cells of the thymus, which are organized in a 3D configuration (111).

By taking advantage from the similarities shared between keratinocytes and thymic epithelial cells (TECs), we used human keratinocytes seeded with human fibroblasts on a 3D poly(ε-caprolactone) scaffold, by mimicking the 3D configuration of the thymus, to verify the ability of these cellular elements, spatially arranged as

described, to reproduce *in vitro* a microenvironment able to replace TECs in supporting HSCs differentiation.

The *ex vivo* reproduction of thymus microenvironment, positively selected $CD34^+$ HSCs (>80%) were seeded in the scaffold and maintained in culture for 5 weeks in the presence of pro-lymphopoietic cytokines, including IL-7 and IL-15, as well as Flt-3 ligand. Subsequently, we performed an immunophenotypic analysis at different time-point to observe developmental changes that occurred when $CD34^+$ HSCs were induced to differentiate in the composites, by using flow cytometry to detect expression of surface antigens critic for T-cell development, such as CD7, CD1a, CD3 and CD8 CD4. Of note, it has been shown that, at that the earliest stages of T-cell development, thymic precursors express CD7 surface antigen (112), since Notch signaling pathway induces the differentiation of multipotent haematopoietic progenitors into $CD7^+CD34^+CD45RA^+$ early T-cell precursors (113). Subsequently, the expression of $CD1a^+$ represents a critical stage to T-cell commitment and the cell population $CD7^+CD1a^+$ has been previously identified as early T cells (114). At the next developmental stage, the pre-T cells express CD4, but not yet CD3 and CD8, and are thought to be CD4 immature single-positive cells. Eventually, the expression CD4 and CD8 is required to generate double-positive, which then mature in single positive CD4 or CD8 cells (115). In the multicellular biocomposite, we observed *de novo* generated $CD7^+$ cells by the third week of culture. At the fifth week of culture, these cells acquired the CD1a marker. In addition, we detected in culture CD4 immature single-positive cells, not yet expressing CD3 and CD8 markers. Taken together, these data demonstrated that in the "thymic organoid", containing skin-derived elements in the absence of thymic stroma, in presence of IL-7, IL-15 and Flt3-ligand, HSCs did start

differentiating and that the process was also directed toward a T-cell lineage commitment. To confirm T-cell lineage specification, the expression levels of selected T-lineage specific genes, including *TAL-1*, *Spi-B*, *RAG1/2* and *PTCRA*, do not expressed in keratinocytes or in fibroblasts, were studied by real-time PCR.

Of note, at early stage of T-cell development, the cells express at high levels many genes (*Sfpi1* (PU.1), *Tal1* (SCL), *Id2*, *Cebpa*, *Bcl11a*) functionally implicated in non-T cell hematopoietic differentiation programs (116, 117, 118), while genes necessary for T-cell gene expression program are downregulated, thus implying a multilineage differentiation potential of early thymocytes. Following, the downregulation of these molecules is strongly correlated with the activation of T-cell gene expression program, whose hallmarks are the expression of *Spi-B*, which is downregulated during the early stages of T-cell development (DN1 and DN2) and upregulated at DN3 stage, and the genes involved in the T-cell receptor re-arrangement, such as *RAG1/2* and *PTCRA* (119, 120, 121). Our data showed both down-regulation of *TAL1* and up-regulation of *Spi-B* in the multicellular composite, consistently with the loss of the multilineage differentiative potential. In addition, *PTCRA* and *RAG2* expression was found at the third week of the culture, suggesting recombination activity. These molecular events were not observed in the control systems and are consistent with the immunophenotypic data, supporting an ongoing process.

In conclusion, our results indicate that, the “thymic organoid”, containing skin-derived elements in the absence of thymic stroma, leads to HSCs differentiation and toward a T-cell lineage commitment in the presence of IL-7, IL-15 and Flt3-ligand, even though fully mature single-positive T cells were not generated in the system.

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Human skin-derived keratinocytes and fibroblasts co-cultured on 3D poly ε-caprolactone scaffold support *in vitro* HSC differentiation into T-lineage committed cells

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Abstract

In humans, the thymus is the primary lymphoid organ able to support the development of T cells through its three-dimensional (3D) organization of the thymic stromal cells. Since a remarkable number of similarities are shared between the thymic epithelial cells (TECs) and skin-derived keratinocytes and fibroblasts, in this study we used human keratinocytes seeded with fibroblasts on the 3D poly ε-caprolactone scaffold to evaluate their ability to replace TECs in supporting T-cell differentiation from human haematopoietic stem cells (HSCs). We observed that in the multicellular biocomposite, early thymocytes expressing CD7$^+$CD1a$^+$, peculiar markers of an initial T-cell commitment, were *de novo* generated. Molecular studies of genes selectively expressed during T-cell development revealed that *TAL1* was down-regulated and *Spi-B* was up-regulated in the cell suspension, consistently with a T-cell lineage commitment. Moreover, *PTCRA* and *RAG2* expression was detected, indicative of a recombinant activity, required for the generation of a T-cell receptor repertoire. Our results indicate that in the multicellular biocomposite, containing skin-derived elements in the absence of thymic stroma, HSCs do start differentiating toward a T-cell lineage commitment. In conclusion, the construct described in this study exerts some properties of a lymphoid organoid, suitable for future clinical applications in cell-based therapies.

Keywords: HSC differentiation, PCL scaffold, skin, T-cell development, thymopoiesis

Introduction

T-cell development is characterized by discrete stage-specific differentiation of haematopoietic progenitor cells occurring within the thymus. At the beginning of the process, haematopoietic stem cells (HSCs) and multipotent or lineage-specific progenitor cells are characterized by the expression of the CD34 marker. In humans, the thymus is the primary lymphoid organ that exerts the unique property of supporting the development of fully mature and self-tolerant T cells (1). The process is intimately linked to the specialized functions of thymic stromal cells (TSCs) and to the thymus architecture (2). Genetic alterations leading to abnormal development of the thymic structures profoundly impair the T-cell differentiation process both in mice and humans (3, 4). An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, which may be epithelial or mesenchymal in origin, through which the developing thymocytes migrate and mature (5). This 3D configuration maximizes the interaction of developing thymocytes with the supporting stromal cells, allowing a proper intercellular cross-talk integral to the development of both T cells and TSCs (6).

A remarkable number of similarities are shared between the epithelial and stromal cells of the thymus and keratinocytes

and fibroblasts of the skin. Both thymic and skin epithelial cells selectively express the FOXN1 transcription factor, which plays a critical role in differentiation and survival of these specialized cells (3, 7, 8). Gene alterations of this transcription factor lead to a complete blockage of the development of cell-mediated immunity also in humans (4, 8, 9). Furthermore, the Notch pathway is shared between thymic stroma and skin elements, where it plays an important role in regulating epidermal differentiation (10). Similarly, within the thymus, the Notch pathway is necessary for T-cell lineage commitment and early stages of thymocyte maturation (11). In addition, keratinocytes express many of the keratins similarly to thymic epithelial cells (TECs) (12). Moreover, human keratinocytes are easier to isolate than TECs. Furthermore, it has been documented that murine TECs can function as epidermal and multipotent hair follicle stem cells when exposed to an inductive skin microenvironment (13). By contrast, a major difference between thymus and skin is the architecture of each organ, in that the skin epithelial cells are mostly distributed along a basement membrane differently from epithelial cells of the thymus, which are organized in a 3D configuration.

The *ex vivo* reproduction of microenvironments of the native tissue through approaches based on the use of porous and biodegradable matrices has been proven useful to repair or replace tissues damaged at a molecular or functional level (14). This approach has been successful in achieving the regeneration of many tissues, such as skin (15), cornea (16), blood vessels (17) and in the bone replacement process (18, 19).

Optimal scaffold materials should exert the properties of excellent biocompatibility, suitable microstructure, controllable biodegradability and suitable mechanical properties to sustain and facilitate a proper intercellular connection (20, 21). Scaffold surfaces need to be organized to allow optimal cell–cell contact, growth, maintenance of morphology and viability over time to meet the demands of the specific application (22).

In congenital immunological disorders and, in particular, in athymic disorders, a scaffold mimicking the 3D structure of primary lymphoid organs may be potentially used for the differentiation of haematopoietic cell precursors and, eventually, allows the re-setting of immunological response through functional or molecular manipulation of precursor cells. In keeping with this, it has been recently documented that a 3D tantalum-coated carbon matrix is able to support the development of functional T cells from haematopoietic precursor cells in the context of a heterogeneous multicellular system (23), even though the capability of the system to ensure a complete process, resulting in mature type T cells, is still under debate (24).

In this study, we verified the hypothesis regarding whether cellular elements of the skin, spatially arranged in a 3D polycaprolactone (PCL) architecture, can support in the absence of thymic components the survival of HSCs and their differentiation into T-lineage committed cells.

Methods

Preparation and characterization of porous scaffolds

The 3D porous scaffolds were developed by adapting the phase inversion and salt leaching technique reported in previous works (25). Scaffold morphology was preliminarily investigated via field emission scanning electron microscope (FESEM; Quanta FEG200, FEI). Specimens were fractured using a razor blade along preferential directions, parallel and perpendicular to the surface. Transverse and longitudinal sections were covered by a thin chromium layer (ca. 20 nm) by automatic sputtering (Emitech K575X) to afford a more efficient electron conductivity of the scaffold surface. The porosity was assessed in terms of pore size, shape and spatial distribution by images at different magnifications and fixed working distance (10 mm). To obtain a quantitative estimation of the scaffold porosity, three different methods were used: weight measurements by a gravimetric method, 2D image analysis (2D-IA) and mercury intrusion porosimetry (MIP). The porosity was obtained by theoretical conditions as previously described (26). The 2D-IA evaluation of porosity features (porosity degree, pore size and spatial distribution) was performed by dedicated software (ImageJ 1.38b; NIH Freeware; National Institutes of Health, Bethesda, MD, USA) (27). The porosity degree was evaluated from the total surface area of counted pores, whereas the pore sizes were derived. Means and standard deviations of pore fraction and size were determined on 10 different scanning electron microscopy (SEM) images. Porosity measurements by MIP were assessed to estimate the really interconnected pores and their specific pore surface. A mercury surface tension of 480 mN m^{-1} and a contact angle of 141.38° were used, while a pressure gradient from 400 Pa up to 200 KPa was intruded to exactly count either micropores or macropores according to the Washburn equation. Means and standard deviations were determined by testing three different samples.

Cell cultures

Normal human epidermal keratinocytes (Clonetics, Lonza) were cultured in supplemented keratinocyte growth medium (KGM-Gold BulletKit, Lonza) as previously described (28). The cultures were incubated at 37°C in an atmosphere supplemented with 5% CO_2, with the cell culture media changed daily. Normal human dermal fibroblasts (Clonetics, Lonza) were grown in fibroblast growth medium (FGM-2 BulletKit, Lonza) and 10% foetal bovine serum (Gibco, Invitrogen Ltd, Paisley, UK), supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Lonza, Basel, Switzerland). The cultures were incubated at 37°C in an atmosphere supplemented with 5% CO_2, with the cell culture media changed daily.

PBMCs were obtained from normal donors of heparinized peripheral blood by Ficoll-Hypaque (Biochrom AG, Berlin, Germany) density-gradient centrifugation. $CD34^+$ HSCs were isolated by incubating $1–2 \times 10^8$ PBMCs in 300 μl of PBE with 100 μl of CD34 (QBEND 10)-conjugated magnetic beads (Multisort beads; Miltenyi Biotec, Bologna, Italy), followed by incubation for 30 min at 4–8°C. After incubation, the cells were washed with ice-cold PBE (PBS/0.5% BSA/5 mM EDTA) and processed through a column placed in a magnetic field and the target cells retained. After washing the column thoroughly with ice-cold PBE, the target cells were recovered by removing the magnetic field and flushing the column with 1 ml of PBE. $CD34^+$ cells were then labelled with

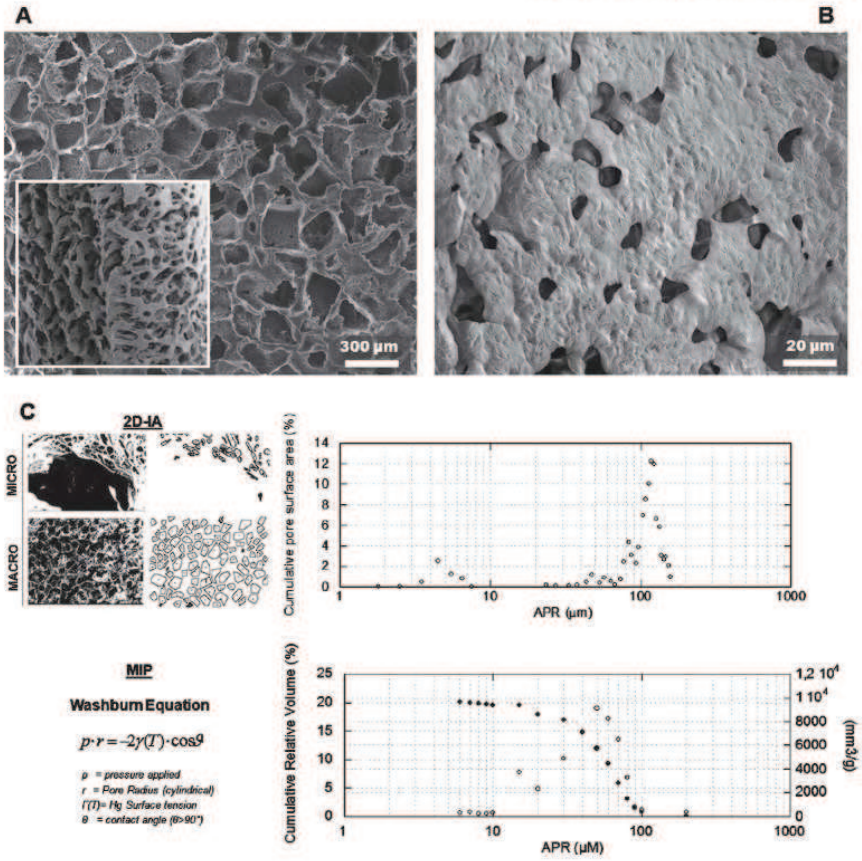

Fig. 1. Qualitative and quantitative investigations of PCL scaffold morphology. Scanning electron micrographs of macropores (cross-section in the square) (A) and micropores (B). (C) Cumulative distribution of pore surface and volume estimated by 2D-IA and MIP techniques, respectively. Scale bar: 20 and 300 µm.

a CD34-FITC-conjugated antibody for 15–20 min at room temperature. The purity of the isolated haematopoietic progenitor cells was evaluated by flow cytometry.

Cell seeding

The PCL scaffolds were incubated in a solution of 100 µg ml^{-1} rat tail collagen I (BD Biosciences, San Josè, CA, USA) in PBS for 30 min at 37°C and rinsed twice in PBS (Cambrex, Charles City, IA, USA). Keratinocytes and fibroblasts were added to each matrix and incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere to allow cell attachment to the composite surface. Non-adherent cells were removed by transferring the matrices to new 24-well plates. Culture medium was added to each matrix and consisted of a 1:1 mix of the fibroblasts and keratinocytes media previously described. The constructs were then cultured for 6 days, and the medium was changed every other day. On day 7, 1×10^5 normal CD34$^+$ HSCs were added to each matrix and the unit was cultured in Iscove's modified Dulbecco's medium (Gibco, Invitrogen Ltd) with 10% heat-inactivated foetal bovine serum (Gibco, Invitrogen Ltd), 20 ng ml^{-1} IL-7 (Invitrogen Ltd), 20 ng ml^{-1} IL-15 (Invitrogen Ltd), 100 ng ml^{-1} Flt3-ligand (Millipore, Billerica, MA, USA) and penicillin/streptomycin. One-half of the medium was aspirated and replaced three times weekly and the culture was maintained for 4–5 weeks.

Proliferation assay

To determine the biological effect of the PCL scaffold on *in situ* cell proliferation, peripheral blood cells were seeded in two different constructs: cell culture in 96-well tissue culture plates (BD Biosciences) in the absence of PCL scaffold and in the presence of PCL scaffold. Moreover, the cells were seeded on the different constructs at a density of 200 000 cells per well and cultured for 4 days in triplicate wells following 8 µg ml^{-1} of PHA (Biochrom AG) stimulation. At the end of the culture period, the cell proliferation was assessed with

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). MTS reagent (20 µl per well) was added at 37°C for 4 h and the light absorbance at 490 nm was recorded using a microplate reader (Epoch multivolume spectrophotometer system). The background absorbance at 490 nm was corrected by subtracting the average absorbance of the control wells without cells from each well to estimate cellular functionality.

SEM analysis

As for the investigation of biohybrid scaffold, cells were fixed for 2 h in 2.5% glutaraldehyde solution and dehydrated with sequential washes in 50, 70, 80, 90 and 100% ethanol. The samples were air dried overnight before the chromium sputtering. In this case, the accelerating voltage of the FESEM equipment (Quanta FEG200, FEI) was set at 5 kV, reducing the vacuum level into the chamber (LV or low vacuum mode), so preventing any negative interaction of electron beam with the cellular bodies.

Flow cytometry

At defined time points (0, 1, 2, 3 and 4 weeks), the cells cultured on each PCL construct were extracted from the scaffolds by aspiration of the medium and flushing of the matrices. Cells were exposed to directly conjugated mouse anti-human monoclonal antibodies to assess HSCs with CD34-APC (BD Biosciences) and CD45-APC-Cy7 (BD Biosciences), and lymphocyte precursors with CD7-PE (Immunological Sciences, Rome, Italy), CD1a-FITC (Dako), CD3-PerCP (BD Biosciences), CD4-PE (BD Biosciences) and CD8-PECy7 (Beckman Coulter). The cells were incubated with directly labelled antibody clones at 4°C in the dark for 30 min, washed and re-suspended in 100 ml of PBS. The events in the displayed graphs and contour plots were gated by forward and side scatter to exclude dead cells. For analysis of early thymocyte subsets with CD7, CD3, CD4 and CD8, T-cell precursors were identified by gating on viable $CD45^{+}$ dim cells. Analytical flow cytometry was performed using a BD FACS Canto II flow cytometer (BD Biosciences). Subsequent data processing and preparation for presentation were done using BD FACSDiva software.

Real-time PCR

The cells cultured on each PCL construct were extracted from the scaffold by aspiration of the medium and flushing of the matrix at different time points. RNA extraction was performed using RealTime ready Cell Lysis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Total RNA was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol using random hexameric primers. Amplification of the complementary DNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche, Branchburg, NY, USA) under the following conditions: 5 min of denaturation at 94°C followed by 55 cycles for 6 s of annealing at 62°C and 5 min of extension at 72°C. Real-time PCR utilized specific primers to amplify *Ikaros*, *TAL1*, *Spi-B*, *PTCRA* and *RAG2* (Table 1). β-*actin* was used as a reference gene. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The ratio of the target gene expression in experimental/control ('fold change in target gene'/'fold change in reference gene') was determined using the ΔΔCt method (29).

Results

Synthesis and characterization of the PCL scaffold

A preliminary evaluation of the PCL scaffold architecture was performed by FESEM microscopy. A typical bimodal porosity, induced by the preparation technique, was obtained as clearly illustrated in Fig. 1(A and B). Larger pores (macropores) ranging from 100 to 300 µm (Fig. 1A) due to the extraction of the sodium chloride crystals were strictly coupled with smaller pores (micropores) ranging from 1 to 20 µm (Fig. 1B) ascribable to phase inversion mechanisms by solvent/non-solvent exchange. The estimation of porosity through gravimetric method revealed a porosity degree of 94.6 ± 0.8%. The porosity value calculated by 2D-IA was 82 ± 4% and underestimated the total macroporosity. As indicated in Fig. 1, this analysis further offered a quantitative estimation of the pore size in terms of the average pore radius (APR) of micropores and macropores. The cumulative distribution of pore surface area (Fig. 1C) clearly evidenced a bimodal population of pores with statistical modes of 117 and 4.5, respectively. In particular, a macropore APR resulted equal to 117.6 ± 11.4, coherently with the average size of the used porogen particles, while a micropore APR equal to 4.5 ± 0.8 is in agreement with the SEM evidence. It is noteworthy that the bimodal porosity represents a key parameter that generally concurs with the interconnection of pores. MIP data confirmed a high fraction of fully interconnected pores, exceeding 90%.

The PCL scaffold does not interfere with the in vitro proliferative capability of PBMCs

To ensure that the scaffold composition did not alter cell physiology in cell culture, we evaluated PBMC proliferation following PHA stimulation. Cell cultures were performed in 96-well tissue culture plates in the absence or presence of a PCL scaffold, for 4 days. The PBMC proliferation in the presence of the scaffold was comparable to that achieved in the absence of the scaffold either in the presence or absence of PHA stimulation (Fig. 2). These results indicate that the PCL scaffold did not interfere with the cellular proliferation, thus indicating that the composite did not have any cytotoxic effect on blood cells.

Table 1. PCR primers for amplification of T-cell lineage-related genes

	Primers	
Genes	Sense	Anti-sense
Ikaros	tcccaagtttcagggaagga	acgactctgtcactcttggagct
TAL1	tctgaagcaaggcggtggac	ggaagaccgtgccgtcttca
Spi-B	tcgccctggaggctgcac	ccccctctgaatcagggta
PTCRA	catcctgggagcctttggt	ccggtgtccccctgagag
RAG2	cctgaagccagatatggtc	gtgcaattcacagctgggct
β-*actin*	gacaggatgcagaaggagat	ttgctgatccacatctgctg

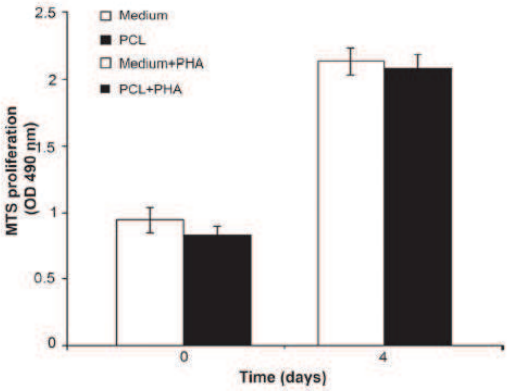

Fig. 2. The PCL scaffold does not interfere with the proliferative capability of blood cells. PBMCs were cultured directly on the composite at a density of 200 000 cells per well. The absorbance was expressed as a measure of the cell proliferation on the PCL scaffold without stimulation or following 8 μg ml^{-1} PHA stimulation for 4 days. Data represent the mean ± SD from three independent experiments performed in duplicate.

The composite provides an appropriate environment to allow an optimal cell–cell interaction by mimicking the 3D configuration of the thymus

To reproduce *in vitro* the thymic microenvironment, previously expanded fibroblasts and keratinocytes were seeded together onto artificial 3D PCL scaffolds. The structure of the composite, after cell seeding and cell infiltration of the PCL scaffold, was studied by SEM of the surface. In such a construct, each cell type was capable of interacting with both the material and with each other, as depicted in Fig. 3. Fibroblasts and keratinocytes adhered and were organized spatially on the surface of inner pores of the material (Fig. 3A). Moreover, as shown in Fig. 3(B), a strong interaction of keratinocytes with fibroblasts occurred, resulting in skin cell occupancy of overlapping sites on the scaffold. In particular, a continuous layer of well attached cells to the upper surface of the scaffold was evident. Fibroblasts grew as adherent cells on the surface of the material, thanks to their stretching filopodi that spanned adjacent matrix components, forming focal adhesions with the matrix (Fig. 3C and D). Figure 3(D) shows in detail the cellular adhesion to the material through thin filopodi. Therefore, this characteristic is most important for our system in that it ensures a stable interaction essential to reproduce an *in vitro* 'organoid'. Furthermore, each cell type was capable of interacting with material (Fig. 3E) and with each other (Fig. 3F) after 3 weeks of culture.

Keratinocytes and fibroblasts seeded on the 3D scaffold support the survival of HSCs and their differentiation into T-lineage committed cells

In this organoid, positively selected CD34$^+$ HSCs (>80%) were seeded in the scaffold and maintained in culture for 5 weeks in the presence of prolymphopoietic cytokines IL-7 and IL-15, as well as Flt-3 ligand. The PCL scaffold without skin elements or culture without the PCL scaffold were used as controls. The cell viability was evaluated by flow cytometric analysis of the pan-leucocyte CD45 marker. Figure 4 shows the behaviour of CD45$^+$ cells in the different composites during the 5 weeks of culture. In the keratinocytes/fibroblasts/PCL scaffold, the survival of CD45$^+$ cells was higher than in the other composites. In particular, in this setting, CD45$^+$ cells represented 70% of cell suspension after 4 weeks of culture, whereas in the control cultures without PCL, no viable CD45$^+$ cells were detected at this time.

To evaluate the importance of the keratinocytes and fibroblasts on the maintenance and differentiation of CD34$^+$ cells, the cells harvested from the constructs were analyzed by flow cytometry during the 5 weeks of culture. As shown in Fig. 5(A) and (B), the CD34$^+$ cells decreased during the cultures. In the keratinocytes/fibroblasts/PCL scaffold, these cells were more represented during the culture and persisted longer than in the control cultures.

Figure 5(C) shows the behaviour of CD34$^+$ HSCs compared with that of CD45$^+$ in the keratinocytes/fibroblasts/PCL scaffold. Differently from HSCs, CD45$^+$ cells persisted in the culture at least for 5 weeks, thus suggesting that in the presence of the multicellular biocomposite, re-arranged in a 3D configuration, stem cells undergo a differentiation process.

An important step in the establishment of an effective *in vitro* system for human T lymphopoiesis is the immunophenotypic characterization of the early stages of T-cell development. To address this issue, we performed a temporal analysis of early developmental changes that occurred when CD34$^+$ HSCs were induced to differentiate in the composites. Of note, CD7 expression is considered to be one of the earliest cell surface markers known to appear during T lymphopoiesis (30). During the development process in the keratinocytes/fibroblasts/PCL biocomposite, the cells expressed the CD7 molecule after 3 weeks of culture (up to 65% of the viable CD45$^+$-gated cells). Interestingly, this expression profile persisted till the fifth week of culture only in the keratinocytes/fibroblasts/PCL scaffold (Fig. 6B). Differently, these early differentiated cells were much less represented in all the control cultures and disappeared by the fourth week. Of note, in the absence of keratinocytes, in spite of the fibroblasts seeded in the 3D scaffold, no CD7$^+$ cells were generated.

The transition from CD7$^+$CD1a$^-$ to CD7$^+$CD1a$^+$ of early thymocytes is peculiar to an initial T-cell commitment. As shown in Fig. 6(C), the CD1a molecule was expressed at the fifth week of culture only on cells cultured in the keratinocytes/fibroblasts/PCL scaffold, accounting for 85% of the viable CD45$^+$-gated cells, differently from the controls. The evaluation of the CD3 expression on cells developed in the *in vitro* system revealed the absence of this marker in the first 5 weeks of culture (Fig. 6D), thus ruling out the presence of contaminating mature T cells. When the expression of CD4 and CD8 markers was evaluated in our system, only the presence of single-positive CD4 cells was detected at the third week of culture in the keratinocytes/fibroblasts/PCL scaffold, accounting for 10% of the viable CD45$^+$-gated cells. Interestingly, these cells persisted until the end of the culture. In the control systems, the CD4$^+$ cells were detected only at the third week, but they disappeared by the fourth week of culture. No single-positive CD8 or double-positive CD4 and CD8 cells were detected (Fig. 6E).

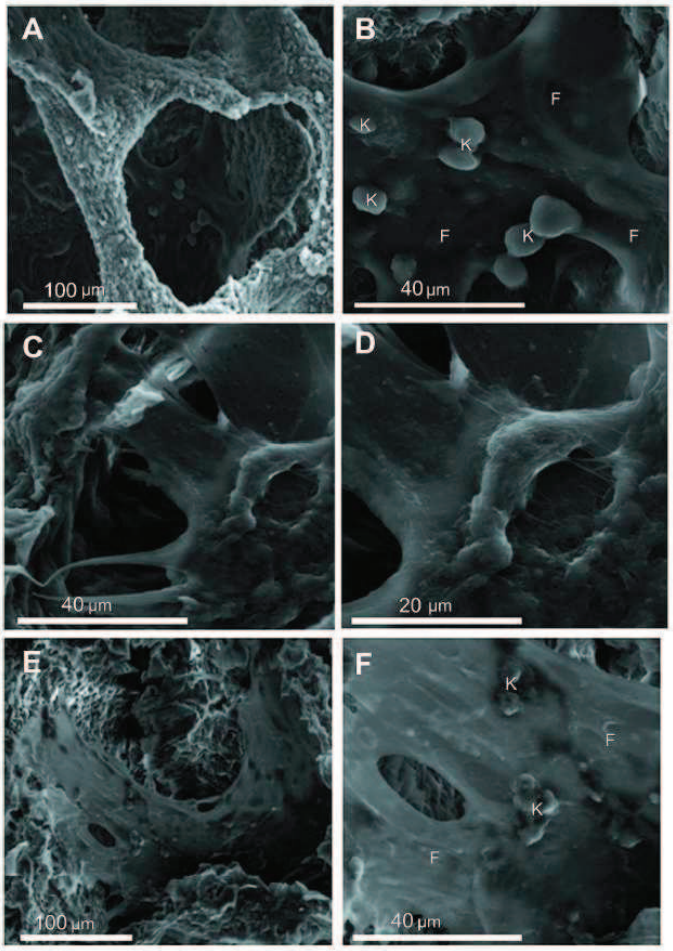

Fig. 3. Representative scanning electron micrographs of keratinocytes and fibroblasts co-cultured on the PCL scaffold. The cells were fixed for 2 h and processed for SEM imaging. Each cell type was capable of interacting with the material and with each other. The panels B, D and F show high-magnification SEM images of the panels A, C and E, respectively. Scale bars: 100 µm (A and E), 40 µm (B, C and F) and 20 µm (D). F, fibroblast, K: keratinocyte. Original magnifications are as follows: panels A and E: ×800; panel F: ×1600; panels B and C: ×3000; panel D: ×6000.

These data suggest that the 3D scaffold colonized with both keratinocytes and fibroblasts promoted HSC commitment to the T-cell lineage as evidenced by the emergence of *de novo* generated $CD7^+$, $CD1a^+$ and $CD4^+$ cells.

To confirm that in the 3D PCL scaffold seeded with keratinocytes and fibroblasts, stem cells underwent to a T-cell linage differentiation process, the expression of selected T-lineage-specific genes, not expressed in keratinocytes or in fibroblasts, were studied by real-time PCR. It is generally agreed that earliest thymic immigrants with T-cell potential also retain the potential to become distinct haematopoietic cell types, such as NK cells, dendritic cells or monocytes. As T-cell precursors differentiate into T cells, they gradually lose the plasticity to become NK or dendritic cells. At this point, the genes required for alternative lineages commitment, such as *TAL1* and *Ikaros* are down-regulated. Simultaneously, T-cell lineage-specific genes, such as *Spi-B*, *PTCRA* and *RAG1/2*, are up-regulated (31). As shown in Fig. 7(A), *Ikaros* expression was only barely detectable in the keratinocytes/fibroblasts/PCL scaffold at the third week of culture in a comparable manner than in the control system without keratinocytes. The relative expression of *TAL1* decreased during the culture in the keratinocytes/fibroblasts/PCL and it was not expressed in the control system (Fig. 7B).

During the T-cell development, *Spi-B* is expressed at a lower extent at an earlier stage and is up-regulated at the

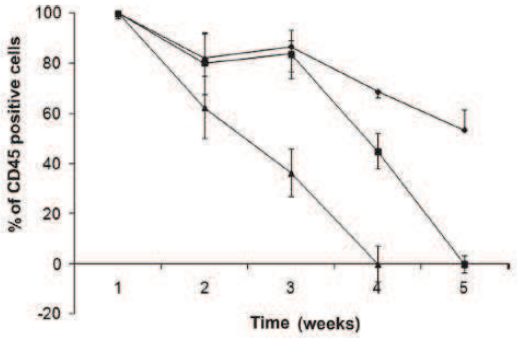

Fig. 4. The multicellular biocomposite supports the survival of HSCs over time in long-term cultures. Percentages of CD45-positive cells in the presence of PCL scaffold/keratinocytes/fibroblasts (closed diamonds), PCL scaffold (closed squares) and medium alone (closed triangles) during the 5 weeks of culture. Measurements were made at 1, 2, 3, 4 and 5 weeks of culture. At these defined time points, the cells were extracted from the PCL scaffold by aspiration of the medium and flushing of the matrix. Data represent the mean ± SD from three independent experiments.

DN3 stage (32). As shown in Fig. 7(C), *Spi-B* was up-regulated at the fifth week of culture in the keratinocytes/fibroblasts scaffold, differently from the control system where *Spi-B* was not expressed at all. These data paralleled the immunophenotypic data of the CD7 and CD1a expression. As for the *PTCRA* gene, it was highly expressed at the third week of culture in the keratinocytes/fibroblasts/PCL scaffold and was down-regulated at the fifth week of culture (Fig. 7D).

RAG2 plays a pivotal role in the initiation of the re-arrangement of antigen-binding domains of the T-cell receptor, being up-regulated at the DN2 stage (33). Only in the keratinocytes/fibroblasts scaffold was *RAG2* expression detectable at either the third and fifth weeks of culture and was comparable to the expression in PBMCs (Fig. 7E). Differently, in the control system, RAG2 relative expression was barely detectable during the culture.

Discussion

In this work, the capability of skin cellular elements, reconfigured in a 3D arrangement, to support the survival of HSCs and their differentiation into T-lineage committed cells has been documented. We observed that, in the absence of thymic cellular epithelial elements, skin-derived keratinocytes and fibroblasts seeded on the PCL scaffold, by mimicking the 3D configuration of the thymus, provide an appropriate environment to allow an optimal cell–cell interaction, able to support HSC commitment to the T-cell lineage.

The 3D organization is unique to TECs, in that in other organs, epithelial cells are 'polarized' and placed on a basal lamina, forming sheets of cells lining internal and external surfaces (34, 35). The peculiar 3D organization of TECs creates a proper microenvironment, which allows thymocyte migration and a tight lymphostromal interaction (5, 36). Foetal TSC monolayer cultures are not able to support T lymphopoiesis, thus indicating that the 3D structure is required (34).

Recently, a thymic organoid has been engineered by seeding a tantalum-coated carbon matrix with thymic murine stroma (37). This composite was able to generate mature functional T cells from bone marrow-derived haematopoietic progenitor cells. In this study, for tissue engineering, a different material, the PCL, has been chosen because it is a biocompatible structure with a high surface area/volume ratio due to its high porosity, thus being an ideal scaffold. In addition, the bimodal population of pores is strongly desired to assure efficient nutrient transport and waste removal within the scaffold. In addition, cell growth and migration are also favoured thanks to a higher surface/volume ratio (38). Our findings are in keeping with previous observations, which indicate that PCL scaffolds are suitable in guiding cell growth and in facilitating the synthesis of extracellular matrix, thus leading to the formation of functional tissues and surrogate organs (39).

Because skin-derived keratinocytes share several similarities with TECs, our data demonstrate that these cells, in the absence of thymic cellular elements, can replace TEC functionality in supporting the T-cell differentiation process. We observed that in the multicellular biocomposite, $CD7^+$ cells were *de novo* generated by the third week of culture. Subsequently, at the fifth week of culture, these cells also expressed the CD1a marker. Furthermore, CD4 immature single-positive cells, not yet expressing CD3 and CD8 markers, were detected during the culture. These findings are in keeping with the observation that thymic precursors, at the earliest stages of T-cell development, first acquire the CD7 marker (30). However, these cells along with T cells can also give rise to NK and myeloid precursors (40). The transition to the $CD7^+CD1a^+$ stage is considered peculiar to a T-cell commitment. At the next developmental stage, the pre-T cells express CD4, but not yet CD3 and CD8, and are thought to be CD4 immature single-positive cells. Later in the differentiation process, the cells express CD4 and CD8 and are referred to double-positive cells that subsequently mature in single-positive CD4 or CD8 cells (41).

In this study, through the evaluation of the expression patterns of genes selectively expressed in the haematopoietic component of the multicellular biocomposite, we confirmed that an *in vitro de novo* generation of cells committed toward the T-cell lineage occurred. Of note, *TAL1* was down-regulated and *Spi-B* up-regulated in the cell suspension, consistently with the loss of the multilineage differentiative potential. Moreover, *PTCRA* and *RAG2* expression was detectable at the third week, indicative of a recombination activity. These molecular events were not observed in the control systems and are consistent with the immunophenotypic data, supporting an ongoing T-cell differentiation process. It is known that at early stages of this process, the cells initially express *TAL1*, which sustains the lineage plasticity. These cells, therefore, retain the potential to become other haematopoietic cell types (42, 43). The down-regulation of this molecule is strongly correlated with the activation of T-cell gene expression program, whose hallmark is the expression of *Spi-B* and the genes involved in the T-cell receptor re-arrangement, such as *RAG1/2* and *PTCRA* (33). Eventually, the cells are committed to a T-cell fate (44).

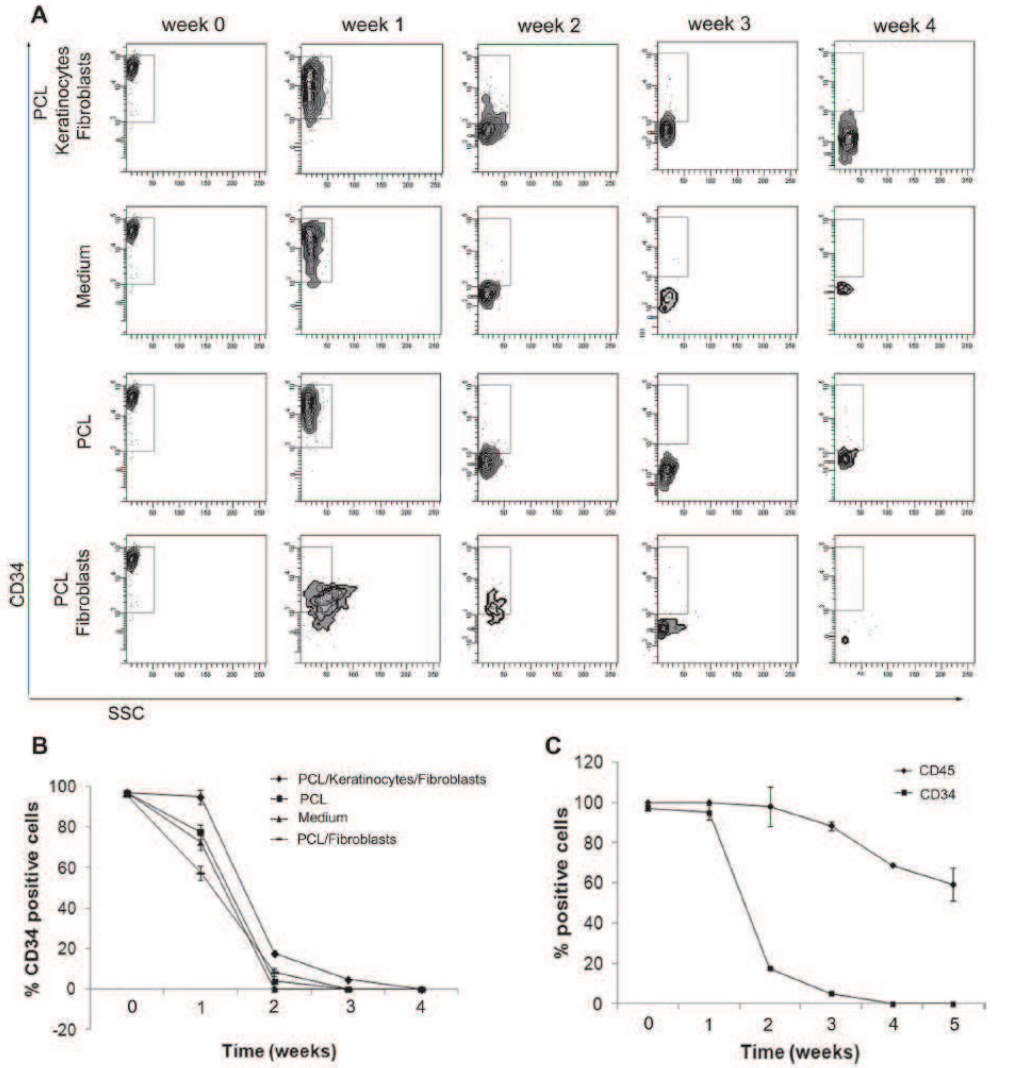

Fig. 5. Keratinocytes and fibroblasts seeded on the 3D scaffold support HSC survival. Representative flow cytometry data for CD34 (gated on CD45$^+$ cells) cell staining (A) and quantification of CD34$^+$ cells cultured on PCL scaffold/keratinocytes/fibroblasts in comparison with the control systems (B). Measurements were made at 0, 1, 2, 3 and 4 weeks of culture. Error bars represent the standard deviation of three independent experiments. (C) Behaviour of CD34$^+$ HSCs and CD45$^+$ cells in the PCL scaffold/keratinocytes/fibroblasts system. Measurements were made at 0, 1, 2, 3, 4 and 5 weeks of culture. Error bars represent the standard deviation of three independent experiments.

Our data, indicating that keratinocytes are able to sustain the process, are not surprising because epithelial and stromal cells of the thymus and skin-derived keratinocytes share a remarkable number of similarities. Of note, keratinocytes express *FOXN1*, a developmentally regulated transcription factor, selectively expressed in epithelial cells of the thymus and skin, where it plays a critical role in cell differentiation and survival resulting in T lymphopoiesis (7, 45). *FOXN1* is also expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation (46–48). Genetic alterations of *FOXN1* lead to athymia (49, 50) and result in humans in a SCID phenotype associated with skin

Fig. 6. Keratinocytes and fibroblasts seeded on the 3D scaffold support HSC differentiation into T-lineage committed cells. (A) Gating strategy for the detection of the investigated cell subsets. (B–E) Representative flow cytometry data for CD45, CD7, CD1a, CD3, CD4 and CD8 staining of cells cultured on PCL scaffold/keratinocytes/fibroblasts in comparison with the control systems at different time points. Numbers indicate the frequency of the cells within the gate. Measurements were made at 0, 1, 2, 3, 4 and 5 weeks of culture. Results shown are representative of at least three independent experiments.

annexa abnormalities, referred as the human equivalent of the mouse nude/SCID syndrome (4, 51, 52). This athymic condition is more severe than that observed in the other athymic conditions represented by the DiGeorge syndrome, which is only characterized by a moderate reduction of T cells, which are in several aspects functional (53). DiGeorge patients, differently from nude/SCID ones, have circulating naive cells, thus suggesting that ectopic thymus anlage or additional structures may contribute to lymphopoiesis. Our data lead us to argue that skin and, in particular, keratinocytes may play such a role thanks to the *FOXN1* expression. Of note, prenatal alteration of the *FOXN1* gene in humans prevents the development of the

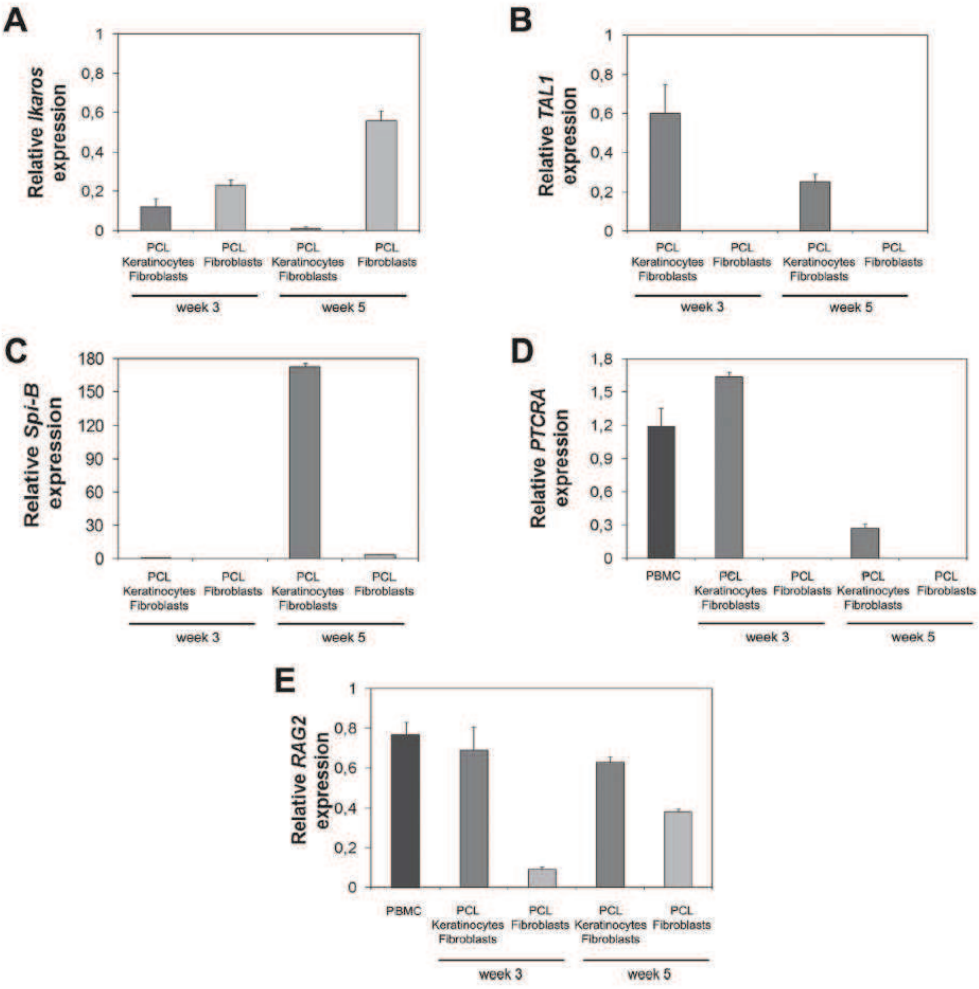

Fig. 7. Expression patterns of selected T-lineage-specific genes during HSC differentiation in the multicellular biocomposite. Real-time PCR evaluation of *Ikaros* (A), *TAL1* (B), *Spi-B* (C), *PTCRA* (D) and *RAG2* (E) expression in suspensions of haematopoietic cells grown on PCL scaffold/keratinocytes/fibroblasts or control systems. Gene expression was normalized to the expression of β-*actin*. The values indicate the relative mRNA expression. Data represent the mean ± SD from two independent experiments.

T-cell compartment, in particular leading to a complete blockage of the $CD4^+$ T-cell lineage maturation (9).

In conclusion, our results indicate that, in a multicellular biocomposite containing skin-derived elements in the absence of thymic stroma, HSCs do start differentiating and that the process is also directed toward a T-cell lineage commitment in the presence of IL-7, IL-15 and Flt3-ligand. However, the maturation process does not lead to the production of fully mature single-positive T cells. This suggests that additional factors or molecular manipulations should be used to reproduce a TEC-like surrogate environment. The *in vitro* re-build of an environment capable of reproducing tissue features of primary lymphoid organs is of valuable help for future therapeutic strategies for patients affected with congenital haematologic and immunologic disorders.

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References

1 Boehm, T. 2008. Thymus development and function. *Curr. Opin. Immunol.* 20:178.
2 Anderson, G. and Jenkinson, E. J. 2001. Lymphostromal interactions in thymic development and function. *Nat. Rev. Immunol.* 1:31.
3 Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. and Boehm, T. 1994. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372:103.
4 Pignata, C., Fiore, M., Guzzetta, V. *et al.* 1996. Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am. J. Med. Genet.* 65:167.
5 Petrie, H. T. 2002. Role of thymic organ structure and stromal composition in steady-state postnatal T-cell production. *Immunol. Rev.* 189:8.
6 Manley, N. R. and Blackburn, C. C. 2003. A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? *Curr. Opin. Immunol.* 15:225.
7 Coffer, P. J. and Burgering, B. M. 2004. Forkhead-box transcription factors and their role in the immune system. *Nat. Rev. Immunol.* 4:889.
8 Romano, R., Palamaro, L., Fusco, A. *et al.* 2012. From murine to human nude/SCID: the thymus, T-cell development and the missing link. *Clin. Dev. Immunol.* 2012:467101.
9 Vigliano, I., Gorrese, M., Fusco, A. *et al.* 2011. FOXN1 mutation abrogates prenatal T-cell development in humans. *J. Med. Genet.* 48:413.
10 Watt, F. M., Estrach, S. and Ambler, C. A. 2008. Epidermal Notch signalling: differentiation, cancer and adhesion. *Curr. Opin. Cell Biol.* 20:171.
11 Radtke, F., Fasnacht, N. and Macdonald, H. R. 2010. Notch signaling in the immune system. *Immunity* 32:14.
12 Laster, A. J., Itoh, T., Palker, T. J. and Haynes, B. F. 1986. The human thymic microenvironment: thymic epithelium contains specific keratins associated with early and late stages of epidermal keratinocyte maturation. *Differentiation* 31:67.
13 Bonfanti, P., Claudinot, S., Amici, A. W., Farley, A., Blackburn, C. C. and Barrandon, Y. 2010. Microenvironmental reprogramming of thymic epithelial cells to skin multipotent stem cells. *Nature* 466:978.
14 Porter, D. L. and Emerson, S. G. 2000. A tissue of T cells. *Nat. Biotechnol.* 18:714.
15 Franco, R. A., Min, Y. K., Yang, H. M. and Lee, B. T. 2013. Fabrication and biocompatibility of novel bilayer scaffold for skin tissue engineering applications. *J. Biomater. Appl.* 27:605.
16 Kadakia, A., Keskar, V., Titushkin, I., Djalilian, A., Gemeinhart, R. A. and Cho, M. 2008. Hybrid superporous scaffolds: an application for cornea tissue engineering. *Crit. Rev. Biomed. Eng.* 36:441.
17 Pankajakshan, D. and Agrawal, D. K. 2010. Scaffolds in tissue engineering of blood vessels. *Can. J. Physiol. Pharmacol.* 88:855.
18 Guarino, V., Causa, F., Taddei, P. *et al.* 2008. Polylactic acid fibre-reinforced polycaprolactone scaffolds for bone tissue engineering. *Biomaterials* 29:3662.
19 Guarino, V., Causa, F. and Ambrosio, L. 2007. Bioactive scaffolds for bone and ligament tissue. *Expert Rev. Med. Devices.* 4:405.
20 Freyman, T. M., Yannas, I. V. and Gibson, L. J. 2001. Cellular materials as porous scaffolds for tissue engineering. *Prog. Mater. Sci.* 46:273.
21 Chen, G., Ushida, T. and Tateishi, T. 2002. Scaffold design for tissue engineering. *Macromol. Biosci.* 2:67.
22 Bramfeldt, H. and Vermette, P. 2009. Enhanced smooth muscle cell adhesion and proliferation on protein-modified polycaprolactone-based copolymers. *J. Biomed. Mater. Res. A.* 88:520.
23 Clark, R. A., Yamanaka, K., Bai, M., Dowgiert, R. and Kupper, T. S. 2005. Human skin cells support thymus-independent T cell development. *J. Clin. Invest.* 115:3239.
24 Meek, B., Van Elssen, C., Huijskens, M. *et al.* 2011. T cells fail to develop in the human skin-cell explants system; an inconvenient truth. *BMC Immunol.* 18:21.
25 Savarino, L., Baldini, N., Greco, M. *et al.* 2007. The performance of poly-epsilon-caprolactone scaffolds in a rabbit femur model with and without autologous stromal cells and BMP4. *Biomaterials* 28:3101.
26 Guarino, V., Causa, F. and Ambrosio, L. 2007. Porosity and mechanical properties relationship in PCL porous scaffolds. *J. Appl. Biomater. Biomech.* 5:149.
27 Guarino, V., Guaccio, A., Netti, P. A. and Ambrosio, L. 2010. Image processing and fractal box counting: user-assisted method for multi-scale porous scaffold characterization. *J. Mater. Sci. Mater. Med.* 21:3109.
28 Ferone, G., Mollo, M. R., Thomason, H. A. *et al.* 2013. p63 control of desmosome gene expression and adhesion is compromised in AEC syndrome. *Hum. Mol. Genet.* 22:531.
29 Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402.
30 Haddad, R., Guimiot, F., Six, E. *et al.* 2006. Dynamics of thymus-colonizing cells during human development. *Immunity* 24:217.
31 Rothenberg, E. V., Telfer, J. C. and Anderson, M. K. 1999. Transcriptional regulation of lymphocyte lineage commitment. *Bioessays* 21:726.
32 Lefebvre, J. M., Haks, M. C., Carleton, M. O. *et al.* 2005. Enforced expression of Spi-B reverses T lineage commitment and blocks beta-selection. *J. Immunol.* 174:6184.
33 Anderson, G., Jenkinson, W. E., Jones, T. *et al.* 2006. Establishment and functioning of intrathymic microenvironments. *Immunol. Rev.* 209:10.
34 Mohtashami, M. and Zúñiga-Pflücker, J. C. 2006. Three-dimensional architecture of the thymus is required to maintain delta-like expression necessary for inducing T cell development. *J. Immunol.* 176:730.
35 Guo, J., Rahman, M., Cheng, L., Zhang, S., Tvinnereim, A. and Su, D. M. 2011. Morphogenesis and maintenance of the 3D thymic medulla and prevention of nude skin phenotype require FoxN1 in pre- and post-natal K14 epithelium. *J. Mol. Med.* 89:263.
36 Petrie, H. T. and Van Ewijk, W. 2002. Thymus by numbers. *Nat. Immunol.* 3:604.
37 Poznansky, M. C., Evans, R. H., Foxall, R. B. *et al.* 2000. Efficient generation of human T cells from a tissue-engineered thymic organoid. *Nat. Biotechnol.* 18:729.
38 Ghosh, S., Viana, J. C., Reis, R. L. and Mano, J. F. 2007. The double porogen approach as a new technique for the fabrication of interconnected poly(L-lactic acid) and starch based biodegradable scaffolds. *J. Mater. Sci. Mater. Med.* 18:185.
39 Rodrigues, M. T., Martins, A., Dias, I. R. *et al.* 2012. Synergistic effect of scaffold composition and dynamic culturing environment in multilayered systems for bone tissue engineering. *J. Tissue Eng. Regen. Med.* 6:e24.
40 Spits, H., Blom, B., Jaleco, A. C. *et al.* 1998. Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol. Rev.* 165:75.
41 Spits, H. 2002. Development of alphabeta T cells in the human thymus. *Nat. Rev. Immunol.* 2:760.
42 Zhang, Y., Payne, K. J., Zhu, Y. *et al.* 2005. SCL expression at critical points in human hematopoietic lineage commitment. *Stem Cells* 23:852.
43 Brunet de la Grange, P., Armstrong, F., Duval, V. *et al.* 2006. Low SCL/TAL1 expression reveals its major role in adult hematopoietic myeloid progenitors and stem cells. *Blood* 108:2998.
44 Rothenberg, E. V. 2007. Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. *Immunity* 26:690.
45 Mecklenburg, L., Tychsen, B. and Paus, R. 2005. Learning from nudity: lessons from the nude phenotype. *Exp. Dermatol.* 14:797.
46 Nehls, M., Kyewski, B., Messerle, M. *et al.* 1996. Two genetically separable steps in the differentiation of thymic epithelium. *Science* 272:886.
47 Itoi, M., Tsukamoto, N. and Amagai, T. 2007. Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus. *Int. Immunol.* 19:127.

48 Bleul, C. C., Corbeaux, T., Reuter, A., Fisch, P., Mönting, J. S. and Boehm, T. 2006. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* 441:992.
49 Schorpp, M., Hofmann, M., Dear, T. N. and Boehm, T. 1997. Characterization of mouse and human nude genes. *Immunogenetics* 46:509.
50 Flanagan, S. P. 1966. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet. Res.* 8:295.
51 Frank, J., Pignata, C., Panteleyev, A. A. *et al.* 1999. Exposing the human nude phenotype. *Nature* 398:473.
52 Pignata, C., Gaetaniello, L., Masci, A. M. *et al.* 2001. Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation. *Blood* 97:880.
53 Gennery, A. R. 2012. Immunological aspects of 22q11.2 deletion syndrome. *Cell. Mol. Life Sci.* 69:17.

1.5. *In vitro* reprogramming of human fibroblasts in medullary Thymic Epithelial Cells (m TECs)

By taking advantage from the similarities shared between keratinocytes and TECs, we used keratinocytes seeded with fibroblasts on a 3D poly(ε-caprolactone) scaffold to replace TECs in supporting HSCs *in vitro* differentiation. Although a T-cell commitment was achieved, we couldn't generate fully mature T cells. To reproduce a microenvironment capable to support a full T-cell differentiation, our further approach was to develop *in vitro* mTECs from human fibroblasts by nuclear reprogramming technology.

Embryonic stem (ES) cells, derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency. The human ES cells, because of their combined ability to proliferate indefinitely and to differentiate in different somatic cells, are suitable for various purposes, including understand disease mechanisms, to screen effective and safe drugs, and to treat patients of various diseases and injuries, such as juvenile diabetes and spinal cord injury. However, ethical controversies does not allow the use of human embryos to derive human ES cells. In addition, it is difficult to generate patient- or disease-specific ES

cells, which are required for their effective application. To circumvent these issues the pluripotent phenotype has been induced in somatic cells by using nuclear reprogramming (122).

Yamanaka and *Takahashi* demonstrated that induced pluripotent stem cells (iPSCs) can be generated from mouse embryonic fibroblasts (MEFs) and adult mouse tail-tip fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely Oct3/4, Sox2, c-Myc, and Klf4 (123). Mouse iPS cells are indistinguishable from ES cells in morphology, ability to proliferate, gene expression, and ability to generate teratoma. Furthermore, when transplanted into blastocysts, mouse iPSCs can give rise to adult chimeras, which are competent for germ-line transmission. These results demonstrated that pluripotent stem cells can be generated from somatic cells by the combination of a small number of factors physiologically expressed in pluripotent cells.

The reprogramming of adult cells is a striking methodology that allows to obtain pluripotent stem cells, but this method have some limitation, including the Currently, the delivery of factors for iPS induction can either be achieved with integrating or non-integrating methods, which differ for safety and efficiency (**Table 2**). The retroviral and lentiviral vectors show high reprogramming efficiency, but the resulting iPSCs have randomly distributed viral transgene insertions, thus increasing tumor formation risk. On the other hand, the transient expression methods, such as adenovirus, plasmid and cell penetrating protein, are of low efficient but they are safe and preserve the genomic integrity of iPS cells (124, 125).

Method of reprogramming	Delivery method	Percentage of publications*	Refs (disease modelling)
Integrating			
Viral[‡]	Retrovirus	76%	37,49,50,59,62-67, 71-73,76,77,95–134
	Lentivirus	20%	38,51,59,61,68–70,72,74, 99,104,131,135–138
Non-viral	Transposon (excisable)	–	–
Non-integrating			
Viral[‡]	Adenovirus	–	–
	Sendai virus	–	–
Non-viral	mRNA	–	–
	miRNA	–	–
	Small molecules	–	–
	Episomal vectors	4%	124,139,140
	Protein	–	–

*Approximate calculation was performed by Pubmed advanced searching on disease modelling studies that used iPS cells over the past 5 years. [‡]National regulations surrounding the use of retroviruses, lentiviruses and Sendai viruses vary among countries (for example, in the USA retrovirally-derived cell lines are considered virus-free after two passages, whereas in parts of Europe these cell lines can never leave bio-safety level II laboratories).

Table 2: Methods of factor delivery for nuclear reprogramming (125).

Human wild-type skin fibroblasts (600000 total cells) have been reprogrammed by introducing 3 episomal vector containing cDNA of human *Oct3/4*, *Sox2*, *Klf4* and *L-Myc* (**Figure 3**) and by using electroporation.

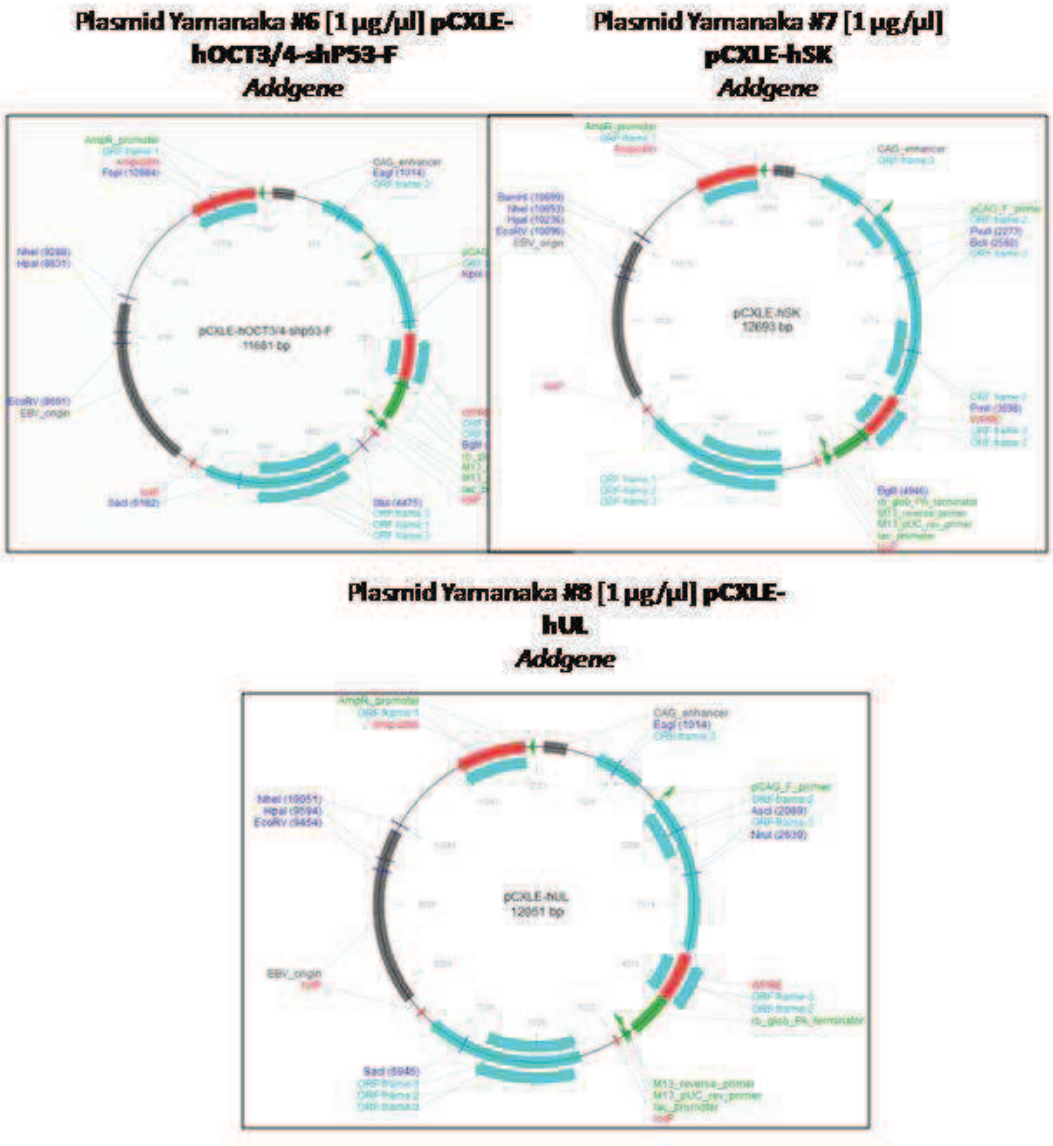

Figure 3: Maps of episomal vector containing of human *Oct3/4*, *Sox2*, *Klf4* and *L-Myc*.

Fibroblasts trasfected have been plated on mitomycin C-treated feeder cells MEFs in a six-well plates, with Fibroblasts Medium Culture without Gentamicin. The next day, the medium has been replaced with new Fibroblasts Medium Culture enriched with Gentamicin. At the 4th day, the medium for primary hES cell culture (hESC medium),

supplemented with FGF2 (10 ng/ml), SB431542 (2μM), PD0325901 (0.5 μM), Valproate (500 μM), has been used. hESCs medium has been replaced every 2 days and the molecule have been added until 14 days after trasfection. ES-like colonies first became visible approximately on 20 days after post-transfection, with a well delimited and tightly packed structure. The cells forming the colonies show large nuclei and scant cytoplasm (Ratio 1/1) (**Figure 4**).

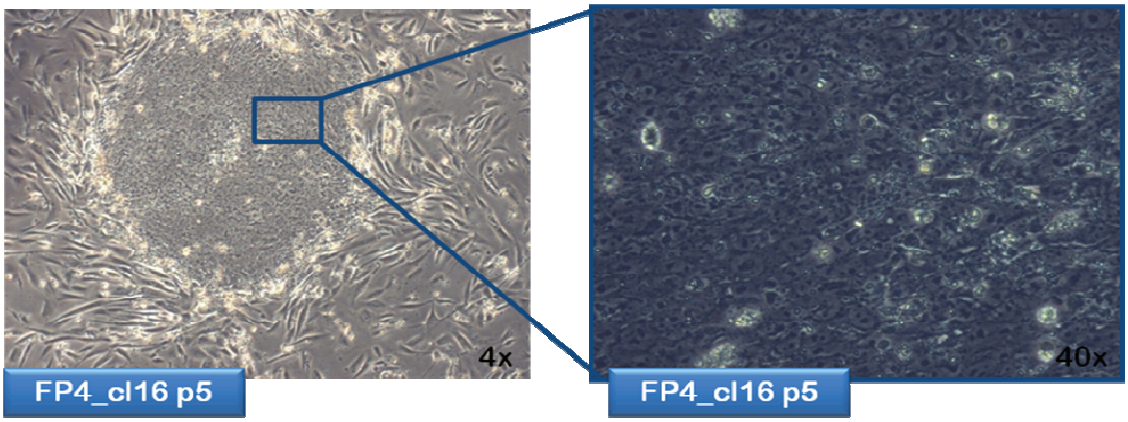

Figura 4: iPSCs clone. A representative clone is showed in the figure. The clone shows the peculiar ES-like clone morphology, namely well delimited and packed, round shape distinct from fibroblasts background, and cells forming the colonies with large nuclei and scant cytoplasm.

The iPSCs clones have been picked until 20 days after trasfection, have been amplified, on feeder layer of BJ1-FGF2 fibroblasts treated with mytomic-C, and freeze by using CryoStor^{TM}CS10 reagent, after 5 passages. It has been obtained 10 iPSCs clones. Moreover, every 2 weeks the mycoplasma test has been performed.

The characterization of iPSC lines, using a standard series of *Quality Control Assays*, is necessary to confirm the pluripotency of iPS clones obtained by reprogramming of somatic cells. The *Quality Control Assays* include: morphology, alkaline phosphatase labeling, pluripotency marker expression, *in-vitro* embryoid body and *in-vivo* teratoma differentiation, karyotype analysis, and eventually the direct differentiation.

Concerning the *Quality Control*, the iPS clones obtained exhibited the peculiar ES-like clone morphology, as showed in the **figure 4**. In addition, the Alkaline Phosphatase staining have been performed on iPSCs clones to proof pluripotent properties of these cells, since Alkaline Phosphatase activity is a qualitative marker of pluripotent cells. The color purple indicate a positive staining for Alkaline Phosphatase, suggesting the presence of pluripotent cells in culture (**Figure 5**).

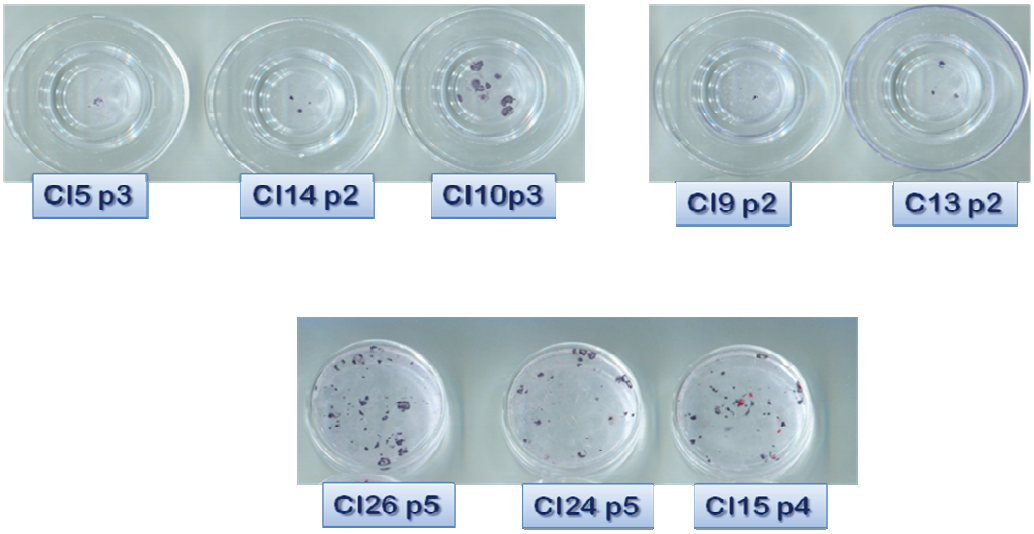

Figure 5: Alkaline Phosphatase staining of iPSCs after passaging. Purple staining identify iPSCs, since high Alkaline Phosphatase can be detected in pluripotent stem cells.

However, additional *Quality Control Assays* have to be performed to provide conclusive evidence of iPSC lines pluripotency.

The future approach will be to differentiate iPSCs into mTECs though the addition of a culture medium enriched with key factors for the differentiation, produced under physiological circumstances during thymic organogenesis in humans. This in vitro model of mTECs will help further clarify the central role of the FOXN1

transcription factor in the Nude/SCID syndrome (the prototype of athymic syndrome and T-cell defect) pathogenesis and explore the possibility to replace TECs with FOXN1-expressing keratinocytes.

1.6. Conclusive remarks

The thymus is the primary lymphoid organ with the unique function to produce and to maintain the pool of mature and functional T cells, through the functions specialized of thymic stromal cells (TSCs) and peculiar 3D architecture, which allows a proper intercellular cross talk. The discovery of disease models associated to mutations of genes implicated in T-cell development, thymus specification and TECs differentiation, provided new and conclusive insights regarding the pathways, the genes, and the molecular mechanism governing these processes and stromal functionality. The studies on both murine and human Nude/SCID phenotype greatly contributed to unravel important issues of the T-cell ontogeny and in particular of the role of *FOXN1* in this process. In spite of the well documented knowledge on the role of the thymus to drive T-cell development, some still unsolved issues in human athymic conditions indicate that an in-depth information of the overall process is still to be achieved and, in particular, the involvement of different tissues in T-cell ontogeny must be definitively clarified.

The development *in vitro* of cellular models of TEC lineage differentiation, by using the technology of nuclear reprogramming, will be certainly useful to better characterize the discrete stages of the TECs differentiation and the molecular mechanism involved in the process. Eventually, the *in vitro* re-build of a thymic

environment capable to reproduce tissue features of primary lymphoid organs (77) could be a promising and valuable tool for the treatment of congenital athymia, including *FOXN1* deficiency, along with the thymus transplantation, which is emerged as a potential treatment for these disorders.

CHAPTER II

"X-linked Severe Combined Immunodeficiency due to mutations of γc"

X-SCID is the most common form of SCID, accounting for approximately half of all cases and it is the main form of $T^-B^+NK^-$, in which T cells and NK cells are absent or profoundly diminished in number, whereas B-cell number is normal. X-SCID is generally fatal unless any treatment aimed to immune system reconstitution. Nowadays, the only treatment for X-SCID is bone marrow transplantation (BMT) from an HLA-matched related donor, which confers to children affected by SCID at least a 70% chance of cure. Moreover, the use of a not fully HLA-matched donor increases the immunologic complication such as graft-versus-host disease (GVHD) associated with a potential long-term decline in immune cell function. X-SCID has recently been successfully treated by gene transfer therapy to hematopoietic stem cells, but serious adverse events have also occurred. In two different gene transfer clinical trials for X-SCID, immunological reconstitution has been observed, thus implying the clinical feasibility of introducing a therapeutic gene into hematopoietic stem cells (126). Unfortunately, leukemia occurred in five patients enrolled in the trials, suggesting that insertional mutagenesis and occurrence of the oncogenesis are adverse effect of gamma-retroviral gene transfer treatment. The discovery of the X-SCID disease gene has led to increased appreciation of the immunologic characteristics of this form of SCID and elucidation of molecular responses of lymphocytes to cytokines.

2.1. The γc-signaling and spontaneous or induced cell proliferation

The cytokine receptors are classified into five families on the bases of extra- and intra-cellular domains structure affinity: the cytokine receptor superfamily, interferon receptor family, tumor necrosis factor (TNF) receptor family, tumor growth factor (TGF), β receptor family and IL-8 receptor family (127). The cytokine receptor superfamily is the largest family, including the receptors for IL-6, IL-11, Oncostatin-M (OSM), Ciliary Neurotrophic Factor (CNTF) and Leukemia Inhibitory Factor (LIF) containing the common gp130 (128), the receptors for IL-3, IL-5 and granulocyte, macrophage colony, stimulating factor (GM-CSF) sharing the common β subunit, and eventually the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 and Growth Hormone Receptor (GH-R) sharing the common γc element (127). The *IL2RG*, localized to chromosome Xq13, encodes a γc transmembrane protein, the transducing component shared by the cytokine receptor superfamily (129). The γc is expressed in lymphocytes and also in other cell types. It is clear that γc cytokines regulate several aspects of immune activation. These molecule plays an important role in supporting survival, proliferation and effector functions of activated immune cells. Of note, regulation of cell survival and cell apoptosis is a delicate teamwork and a balanced act of all γc-dependent cytokines is of crucial importance (**Figure 6**) (130).

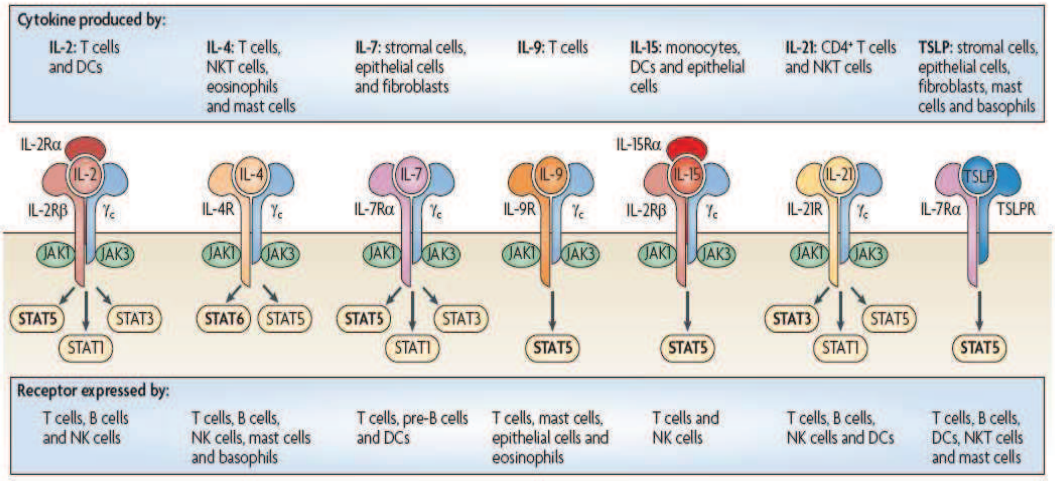

Figure 6: The γc-dependent cytokines and their receptors (131).

The biological effects of cytokines are mediated through interaction with specific receptors, that leads to phosphorylation of intracellular proteins. Members of the cytokine receptor superfamily do not have intrinsic kinase activity, but the interaction with the specific ligands induces the recruitment of intracellular protein kinases. The tyrosine kinases that couple extracellular cytokine binding to intracellular phosphorylation of protein substrates, and eventually to cell growth and differentiation, are members of the Janus-associated kinase (JAK) family. Thus far, four distinct members of the JAK family are known in humans: JAK1, JAK2, JAK3 and Tyk2. Members of the *IL2RG* superfamily physically are associated with JAK1 and JAK3 (132).

Several signaling pathways are elicited by JAK1/JAK3 activation in members of the cytokine receptor superfamily. First, the phosphorylated cytokine receptor may associate with the adaptor SHC, which is itself phosphorylated and binds to Grb2. Grb2 may thus anchor to Sos, the Ras guanine nucleotide exchanging factor. Membrane translocation of the Grb2/Sos complex catalyzes the conversion of inactive GDP-bound Ras to the active GTP-bound state, leading the activation of Raf-1 mitogen-activated

protein kinase (MAPK) and eventually in the induction of immediate-early genes (c-fos, c-jun) (133). Subsequently, the JAK activation by γc-dependent cytokines leads to the binding and phosphorylation of insulin receptor substrates (IRSs) (134). The activation of IRS induces the binding of its SH2 domain to the p85 subunit of phosphatidylinositol-3-kinase (PI3K) and the catalytic activity of the p110 subunit of PI3K. In addition, to promote PI3K activation, IRS recruits Grb2 to allow an increased Ras/raf-1 signaling pathway. Following, to elicit a proper JAK signaling transduction, the phosphorylation of the class of transcription factors known as signal transducers and activators of transcription (STATs) is required. The STATs family comprises a group of cytoplasmic proteins, involved in several functions, including the regulation of the gene expression, cell differentiation, survival and apoptosis. Seven mammalian STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) have been characterized, so far (135). The STAT proteins, containing SH2 and SH3 domains, may undergo JAK-mediated phosphorylation in a tyrosine residue. Following cytokine interaction with receptor and triggering of the JAK-mediated signaling pathway, STATs may interact with the cytokine-receptor complex by binding via their SH2 domain to the phosphotyrosine of the cytokine receptor chain. In addition, following STATs phosphorylation, homo- or hetero-dimerization occurs, by binding of the SH2 domain of one STAT molecule to the phosphotyrosine of the second STAT. The specificity of the response to cytokines is largely dependent on the particular combination of STATs recruited by the different signal-transducing chains of the cytokine receptor. The differences in the STATs binding residues of the various cytokine receptors result in recruitment of specific STATs. The dimerization of STATs induces translocation of the

proteins into the nucleus, where they bind to consensus sequences in the enhancer elements of the promoter regions of target genes, leading to gene transcription.

A potential role of γc in GH-R signaling has been proposed on the basis of the impairment of various GH-induced events in γc deficient conditions. First, the signal transduction properties of GH-R in B-cell lines from X-SCID patients, following GH stimulation, is abnormal, in that GH stimulation fails to induce phosphorylation on tyrosine residues of several proteins, including STAT5 molecule (136). Previously, it has been reported on a patient affected with X-SCID, short stature and peripheral GH hypo-responsiveness, an abnormal protein phosphorylation that normally occurs following GH-R stimulation. Of note, in this patient the immunological reconstitution through bone marrow transplantation paralleled the restoring of GH-R functionality, which resulted in a normal production of Insuline Growth Factor-I (IGF-I). GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function. GH-R consists of a transmembrane protein that contains two motifs and an extracellular domain (137). It remains to be further elucidated whether the γc involvement is required for the expression of the biologic effects of GH and its intermediate molecules on cell growth in either physiological or pathogenic conditions.

The ability of γc to regulate cell cycle progression has been long debated. The gene therapy trials have been proved as an efficace alternative approach to cure X-SCID patients carrying mutations of γc, but the lymphoproliferation documented in 5 out of 20 patients enrolled into the two different trials (138), suggested that retroviral insertional mutagenesis could occur and play a role in oncogenetic transformation. In 2 cases it was associated with an insertional mutagenesis in *LMO2* oncogene (139). Of note,

lymphoproliferation event has not been observed in gene therapy trials for SCID due to ADA deficiency (140), raising the possibility that γc could be oncogenic *per se*.

In keeping with this hypothesis, overexpression of γc transduced through a lentiviral vector into stem cells in murine model of X-SCID led to T-cell lymphomas and thymic hyperplasia in a third of the cases. Intriguingly, no common integration site was found between the mice, which developed T-cell lymphomas. In these mice, differently from humans treated with gene therapy for X-SCID, the expression levels of the protein was elevated, thus implying that the amount of the protein may be crucial for the γc control of cell cycle. These results suggest that insertional mutagenesis may not be the only cause of leukemogenesis and that the expression levels of γc could influence the cell cycle progression directly or its effect being mediated by cytokines signaling pathway.

On the light of the previous observations, to define an intrinsic mitogenic property of γc dependent on the amount of the protein, we used *in vitro* cellular models containing different amounts of γc. In particular, EBV-transformed B-cells (BCLs) from normal subjects, cells transduced with lipid vector containing non-targeting short interfering RNA (siRNA), BCLs transduced with siRNA to knockdown γc expression and BCLs from X-SCID patients were used. Our results indicate that silencing of γc induces a substantial decrease of protein amount in BCLs, demonstrating a direct involvement of γc in self-sufficient growth of BCLs in a concentration dependent manner. We, also, found that the amount of constitutively activated JAK3 parallels the extent of γc expression. This finding is intriguing, in that constitutively active or hyperactive JAK proteins have crucial roles in hematopoietic malignancies, by promoting oncogenic transformation (141). In particular, JAK overexpression can be considered as one of the main biologic events leading to the constitutive activation of

the JAK/STAT pathway that contributes to oncogenesis. In lymphoid cells, the involvement of the JAK/STAT pathway in several cellular processes, such as proliferation and protection from apoptosis, has also been well documented (142). We found that γc silencing also inhibits GH-induced cell proliferation. In this context, it is known that the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines and STAT5 is considered a transforming agent in lymphoma and other cell types (143), therefore we found that the reduction of γc amount also inhibits STAT5 activation and its subsequent nuclear translocation, which follows GH-R perturbation. Of note, it should be mentioned that experimental studies document a role for GH in the initiation and/or promotion of tumorigenesis, raising the possibility that patients treated with GH might be at increased risk of cancer (144). Moreover, a putative role of GH as a cofactor in tumor growth is plausible, since several carcinomas express GH-R (145). In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events (146). In conclusion, these data demonstrate a direct relationship between the amount of γc expression and its role in cell cycle progression. These data add new evidence for a possible intrinsic mitogenic role of γc related to its cellular amount. This biologic effect could be direct, thus related to the molecule *per se*, or indirect and mediated by the participation to cytokine-receptors signaling.

2.2. The γc promotes cell cycle progression and survival in human continuous malignant cell lines

Tumor cells exhibit the aberrant ability to proliferative without control due to mutations affecting genes involved in cell cycle progression, a tightly regulated process (147). Moreover, the impairment of apoptosis is a critical step in cancer development (148, 149), and BCL-2 family proteins play a pivotal role in promoting tumor cell survival (150, 151). Cytokines that signal through receptors sharing the γc lead to transition into the cell cycle and thus proliferation (131). The entry of cells into the cell cycle is controlled by an ordered expression/activation of cyclins (152). In addition IL-2R, through γc, appears to activate a variety of downstream signaling pathways that converge on the regulation of Bcl-2, including PI3K and MAPK activation and transcription of the c-myc gene (153). In turn, c-myc cooperates with STAT5 to induce the expression of cyclin D-type and to promote proliferation (154, 155, 156).

In the light of these observations, we studied the involvement of γc in these important biological process crucial for tumor progression and growth, to verify its direct role in tumorigenesis.

To determine whether γc deficiency had an effect on cell survival we examined BCLs from healthy donors and X-SCID patients. The percentage of live cells was determined using trypan blue staining in the absence or presence of anti-Fas to trigger programmed cell death. In unstimulated X-SCID BCLs there was an increase in the percentage of cell death. Following stimulation with anti-Fas, control and X-SCID BCLs showed a higher and comparable degree of cell death. Programmed cell death is mainly mediated by activation of several caspases (157). These molecules exist as pro-forms that are activated by cleavage by the upstream caspase in the cascade (157). In

unstimulated X-SCID BCLs the low viability was not a caspase-dependent process, since the presence of the cleaved protein was observed only following anti-Fas stimulation. Caspase-independent cell death has been attributed to mitochondrial damage (158), which can be regulated by Bcl-2 family members (159, 160). Bcl-2 and Bcl-XL operate as critical components in a complex network to integrate information and make ultimate life/death decisions. Since the γc-dependent cytokines promote cell survival by up-regulating the antiapoptotic factor Bcl-2 (161) and Bcl-XL (162), the expression of Bcl-2 and Bcl-XL in control and X-SCID BCLs was evaluated. In γc-deficient cells, the expression of Bcl-2 and Bcl-XL was greatly decreased as compared with the control. These findings indicate that γc is required for cell survival and is dispensable for Fas induced cell death. Moreover, the evaluation of molecular expression of BCL-XL in unstimulated cells through quantitative real-time PCR, revealed that in the X-SCID cells, BCL-XL mRNA was 35% of the control. However, evidence exists that autophagy can plays an active role in cell death, by contributing to cell death in unfavorable settings such as nutrient or growth factors deprivation (163). In keeping with this, probably γc could have a role in the autophagy process.

Subsequently, to define whether the effect of γc on cell cycle progression is a peculiarity of lymphoblastoid cells or a more general phenomenon involved in cell growth of malignancies of hematopoietic cell lineages, we evaluated whether γc expression could interfere in cell cycle progression in neoplastic cells. We first examined the expression of the molecule in cell lines obtained from hematopoietic tumors, such as Molt-4, Raji, Rj225 and K-562. The protein was expressed predominantly in the K-562 and to a lesser extent in the Molt-4, Raji and Rj225 in a decreasing order. In X-SCID BCLs, γc expression was completely undetectable. The

expression of γc was also evaluated in primary leukemic samples from patients with ALL, by quantitative real-time PCR. *IL-2Rγ* mRNA levels was greatly increased in leukemic cells as compared with the control. Moreover, to determine whether the expression levels of γc correlated with the self-sufficient growth in malignant cell lines, we examined the proliferation activity of cells under serum-deficient conditions. This was first evaluated by comparing the CFSE dilution profile, upon trypan blue exclusion assay, of several malignant cells. After 5 hours of serum-free culture, some variations in the rate of proliferation among the lines were already evident. K-562 had a high proliferation rate, compared with the other cell lines, while no proliferation was observed in X-SCID BCLs. Moreover, the proliferation of these cell lines was also assessed by 3H-thymidine incorporation assay. The data were in keeping with the results of CFSE experiments and a statistically significant relationship between γc expression and spontaneous cell growth was documented in the examined cell lines ($R = 0.98$, $p < 0.05$).

In the light of these findings, we hypothesized that the inhibition of γc expression in hematopoietic malignant cell lines might have a direct effect on proliferation of these cells. Short interfering RNA (siRNA) was used to knockdown the molecule in these cell lines. Efficiency and specificity of targeted siRNA sequences were confirmed by Western Blot analysis on total lysates and quantitative real-time PCR on mRNA. The results of western blot assay revealed that at 96 hours following the transfection, cells transduced with siRNA had less γc protein than the correspondent cells transduced with the control negative siRNA. In addition, a decrease of the *IL-2Rγ* mRNA was observed in all cell lines, revealing a knockdown efficiency of approximately 85%. In X-SCID cells, *IL-2Rγ* mRNA was undetectable. Moreover, γc

knockdown led to a significant decrease of proliferation. In particular, γc-silencing reduced cell proliferation of Rj225, K-562, Molt-4 and Raji, as compared with control siRNA cells. Taken together, these data confirm that γc plays a key role in the proliferation of these malignant cell lines.

Alterations in cell cycle machinery, mainly in the regulation of G1/S phase, are known to be associated with the development of solid tumors as well as hematological malignant diseases (164). To examine the mechanisms by which γc regulates cell cycle progression, we examined whether different amounts of the molecule were able to influence the transcription of genes selectively involved in cell cycle. Of note, cyclins are the key regulators of cell cycle progression (152). In particular, during the G0 to G1 phase transition, cyclins D1, D2 and D3 are the first molecules to be induced. Cyclin A2 gets activated during the transition from G1 to S phase and B type cyclins are detected during G2 exit and mitosis phase (165). Namely, cyclins A2 and B1 have been implicated in the pathogenesis of cancer and are overexpressed in several tumors (166, 167). Evidence indicates that these cyclins are key components of the cell-cycle machinery (168) and, in particular, cyclin A is expressed at high levels in hematopoietic stem cells and is essential for their proliferation (169). In our study, we observed that the expression of A2 and B1 cyclins strongly paralleled the proliferative capability of malignant cell lines. Interestingly, a positive correlation between the amount of γc and the expression of cyclins A2 and B1 was also found. Taken together these data indicate that the higher is the rate proliferation of a certain cell line the higher is the expression of both γc and cyclins A2 and B1, thus confirming their involvement in the process in a concentration dependent fashion. We also found an increased expression of all D-type cyclins in those cell lines that proliferated mostly, K-562 and Molt-4, whereas they were

not expressed in the other cell lines, but D1 found in Rj225. D-type cyclins are strongly expressed in many malignancies. Overexpression of cyclin D1 protein was documented in many forms of cancer, including breast cancer (170), while overexpression of cyclin D2 was noted in a wide range of B cell malignancies, such as B cell chronic lymphocytic leukemia (171). Like the other D cyclins, cyclin D3 is rearranged and the protein is overexpressed in several human lymphoid malignancies. It was documented that knockdown of cyclin D3 inhibits the proliferation of acute lymphoblastic leukemia cells (172). However, while A and B type cyclins seem to be vital and necessary components of cell cycle progression (169), D-type cyclins may be dispensable for proliferation under certain circumstances, in that different cell types are sensitive to cyclin D knockdown at a different extent (173). This would suggest that they regulate cell cycle in a cell-type specific manner and that there are alternative mechanisms allowing cell cycle progression in a cyclin D-independent fashion (173). Anyway, a critical role for oncogenic transformation of D-type cyclins is a well established feature. In conclusion, these data indicate that γc is strongly implicated in cell cycle progression of hematopoietic malignancies in a similar fashion to the role played in control lymphoblastoid cells, as previously described. This biologic effect is strictly dependent on the expression level of the molecule and can be abolished by gene knockdown. Of note, a direct correlation between the amount of γc expression and the proliferative capability of the malignant cell lines and the regulatory elements of cell cycle progression, A and B cyclins, was found, implying the direct involvement of the molecule in this biological process.

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Role of the common γ chain in cell cycle progression of human malignant cell lines

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Abstract

The γ-chain (γc) is a transducing element shared between several cytokine receptors whose alteration causes X-linked severe combined immunodeficiency. Recently, a direct involvement of γc in self-sufficient growth in a concentration-dependent manner was described, implying a direct relationship between the amount of the molecule and its role in cell cycle progression. In this study, we evaluate whether γc expression could interfere in cell cycle progression also in malignant hematopoietic cells. Here, we first report that in the absence of γc expression, lymphoblastoid B-cell lines (BCLs) die at a higher extent than control cells. This phenomenon is caspase-3 independent and is associated to a decreased expression of the antiapoptotic Bcl-2 family members. By contrast, increased expression of γc protein directly correlates with spontaneous cell growth in several malignant hematopoietic cell lines. We, also, find that the knockdown of γc protein through short interfering RNA is able to decrease the cell proliferation rate in these malignancies. Furthermore, an increased expression of all D-type cyclins is found in proliferating neoplastic cells. In addition, a direct correlation between the amount of γc and cyclins A2 and B1 expression is found. Hence, our data demonstrate that the amount of the γc is able to influence the transcription of genes involved in cell cycle progression, thus being directly involved in the regulatory control of cell proliferation of malignant hematopoietic cells.

Keywords: cell proliferation, cytokines, gamma chain, SCID

Introduction

The common γ-chain (γc) gene localized to chromosome Xq13 encodes a transmembrane protein which is a transducing element shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (1). Deficiency in the expression or function of the γc causes the X-linked severe combined immunodeficiency (X-SCID), characterized by the complete absence of both T and NK lymphocytes and normal B-cell number (2). It is known that the cytokines that act through the γc are generally growth factors (3). Upon cytokine interaction, IL-2R activates numerous downstream signaling molecules, including Janus kinases (JAK) and signal transducers and activators of transcription (STAT). In particular, STAT5, which is directly phosphorylated and activated by JAK3 (4), seems to play a major role in cell proliferation (5–7). γc signaling cytokines promote proliferation in T-cell acute lymphoblastic leukemia (ALL), thus implying a synergistic effect of these cytokines on tumor growth (8). It was also reported that *IL2RG* cooperates with *LMO2* in inducing hematopoietic tumors in X-SCID patients enrolled in gene therapy trials (9, 10).

Recently, we documented a direct relationship between the amount of γc expression and its role in cell cycle progression. In particular, using lymphoblastoid B-cell lines (BCLs), we described a direct involvement of γc in self-sufficient growth in a concentration-dependent manner. Furthermore, the γc amount correlated with the amount of constitutively activated JAK3, while a reduction of γc protein expression resulted in reduced STAT5 nuclear translocation in BCLs (11). This finding led to hypothesize that γc, through the modulation of genes related to growth signaling and cell cycle control, under certain circumstances plays a role as potential oncogene in tumor growth. However, whether γc is implicated in anti-apoptotic mechanism or directly involved in promoting cell signaling of cell cycle progression is not clear.

Cell cycle progression is a highly organized and regulated process that controls cell proliferation (12). Cytokines that signal through receptors sharing the γc lead to transition into the cell cycle and thus proliferation (3). The entry of cells into the cell cycle is controlled by an ordered expression/activation of

cyclins (13). IL-2R through γc appears to activate a variety of downstream signaling pathways that converge on the regulation of Bcl-2 (6), including PI3K and MAPK activation (14) and transcription of the *c-myc* gene (6). In turn, *c-myc* cooperates with STAT5 to induce the expression of cyclin D and to promote proliferation (15–17). It is clear that alterations in Bcl-2 levels exert potent effects on cellular survival and, namely, Bcl-2 overexpression can be tumorigenic (18). Moreover, γc is required for a wide range of signaling inputs that induce cell proliferation through cyclin D3 expression (19).

To define whether the effect of γc on cell cycle progression is a peculiarity of lymphoblastoid cells or a more general phenomenon involved in cell growth of malignancies of hematopoietic cell lineages, in this study, we evaluated whether γc expression could interfere in cell cycle progression in neoplastic cells. We also investigated whether the effect of γc is mediated by A2, B1, D1, D2 and D3 cyclins and is required for a proper activation of these cyclins in cell cycle progression.

Methods

Cell cultures

PBMCs were obtained from X-SCID patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation (20). Lymphoblastoid B-cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMC using standard procedures. The human T-ALL cell line (Molt-4), the chronic myelogenous leukemia cell line (K-562), Burkitt lymphoma cell line and its isogenic derivatives (Raji and Rj225) were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, California), 2 mmol L^{-1} L-glutamine (Gibco), and 50 μg ml^{-1} gentamycine (Gibco) and cultured at 37°C, 5% CO2. Serum starvation was used to synchronize tumor cells in the G0/G1-phase of the cell cycle. The cells were incubated in medium without FBS for 24 h. In self-sufficient growth experiments, cells were cultured in DMEM/F12 (Lonza) without FBS and supplemented with 2 mmol L^{-1} L-glutamine.

Primary leukemic cell lines, consisting of ALL cells, were obtained from aspirated bone marrow of three patients. Normal bone marrow cells were obtained from healthy donors and used as control cells.

Cell proliferation assays and cell survival

Cells were plated in triplicate at 1×10^5 viable cells/well in 96-well plates (BD Biosciences, San Jose, CA), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine for 8 h before harvesting and the incorporated radioactivity measured by scintillation counting. Cell proliferation was also analyzed by the CFSE dilution assay. Cells (1×10^6) were resuspended in 1 ml PBS-10% FBS and labeled with 1.7 μM CFSE (Molecular Probes, Leiden, Netherlands). After 2 min in the dark at room temperature, cells were washed in FBS and PBS. After 6 h, cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Cell viability was determined using trypan blue staining. Cell survival was evaluated following stimulation with anti-Fas mAb (400 ng ml^{-1}; Upstate, Lake Plasid, NY) for 6 h.

Western blot analysis

Stimulated or unstimulated cells were washed with ice-cold PBS (Cambrex, Charles City, IA) and lysed in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na3VO4), 5 μg ml^{-1} leupeptin and 5 μg ml^{-1} aprotinin on ice for 45 min. The cell lysates were stored at −80°C until processing. Proteins were separated on 12% SDS-PAGE. The membranes were then washed three times in wash buffer and incubated overnight at 4°C with the specific primary Abs for IL-2Rγ (Santa Cruz, Santa Cruz, CA), caspase-3 (Cell Signaling, Danvers, MA), Bcl-2, Bcl-XL and beta-actin (Santa Cruz). Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked Abs. ECL kit (Amersham Biosciences, Brussels, Belgium) was used for visualization.

Densitometric analysis was performed after background equalization through the ImageJ software.

siRNAs and transfection

The validated chemically modified oligonucleotides used as short interfering RNA (siRNA) for IL2RG or random non-silencing nucleotides with no known specificity siRNA, used as negative control, were obtained from Invitrogen (Paisley, UK). These siRNAs were transfected at a concentration of 200 pmol/1×10^6 cells in a six well plate for 96 h. The transfection was performed by the lipid vector Lipofectamine 2000 kit (Invitrogen), according to the manufacturer's instructions. Preliminary experiments were performed to establish the silencing efficiency by testing two different oligonucleotides obtained from Invitrogen. The amount of protein expression reduction was calculated as follows: $1 - (OD_{siRNA} \times 100/OD_{control\ siRNA})$.

Real-time quantitative reverse transcriptase PCR analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA was reverse transcribed in the presence of SuperScript II RT (Invitrogen) and oligo(dT) primers (Invitrogen) at 50°C for 50 min and then at 85°C for 5 min to inactivate the enzymes. Amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche, Branchburg, NY). Primers are listed in Table 1. The PCR conditions comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles at 62°C for 20 s and 72°C for 5 min. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The results were normalized to beta-actin. The relative levels of gene expression are represented as $-\Delta Ct = (Ct_{gene} - Ct_{reference})$ and the fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (where Ct is cycle threshold), as previously described (21).

Statistical analysis

MedCalc for Windows was used to analyze the data. The correlations between thymidine incorporation and γc expression and between cyclins and γc expression were obtained using the Pearson's correlation. $P < 0.05$ values were considered statistically significant.

Table 1. Primers used for real-time qRT-PCR

Gene	Primers sequence 5′-3′
Bcl-XL	5′-GTAAACTGGGGTCGCATTGT-3′ 5′-TGCTGCATTGTTCCCATAGA-3′
Cyclin D1	5′-AGGTCTGCGAGGAACAGAAGTG-3′ 5′-TGCAGGCGGCTCTTTTTC-3′
Cyclin D2	5′-CTGTGTGCCACCGACTTTAAGTT-3′ 5′-GATGGCTGCTCCCACACTTC-3′
Cyclin D3	5′-GCAGCGCCTTTCCCAACT-3′ 5′-TCAAAAGGAATGCTGGTGTATGTATC-3′
Cyclin A2	5′-CTGCTGCTATGCTGTTAGCC-3′ 5′-TGTTGGAGCAGCTAAGTCAAAA-3′
Cyclin B1	5′-CGGGAAGTCACTGGAAACAT-3′ 5′-AAACATGGCAGTGACACCAA-3′
IL-2Rγ	5′-TGCTAAAACTGCAGAATCTGGT-3′ 5′-AGCTGGGATTCACTCAGGTTTG-3′
Beta-actin	5′-GACAGGATGCAGAAGGAGAT-3′ 5′-GACAGGATGCAGAAGGAGAT-3′

Results

Deficiency in the expression of γc impairs cell survival

γc-dependent cytokines have important functions related to cellular survival (3). To determine whether γc deficiency had an effect on cell survival, we examined BCLs from healthy donors and X-SCID patients. The percentage of live cells was determined using trypan blue staining in the absence or presence of anti-Fas to trigger programmed cell death (22). In unstimulated X-SCID BCLs, there was an increase in the percentage of cell death, accounting for 25% as compared with 7% of control BCLs. Following 6 h of stimulation with anti-Fas, control and X-SCID BCLs showed a higher and comparable degree of cell death (32 and 36%) (Fig. 1A). Data on BCLs viability was confirmed by PI staining (data not shown). The increase of cell death observed is directly related to the lack of γc expression, since the neutralization of γc-dependent cytokines, as IL-2 or IL-4, did not modify the effect of γc on cell viability of normal cells (data not shown).

Programmed cell death is mainly mediated by activation of several caspases (23). These molecules exist as proforms that are activated by cleavage by the upstream caspase in the cascade (23). Western blot analysis with antibodies to the cleaved/activated form of caspase-3 revealed the presence of the cleaved protein only following anti-Fas stimulation (Fig. 1B), thus indicating that in unstimulated X-SCID BCLs the low viability was not a caspase-dependent process. Caspase-independent cell death has been attributed to mitochondrial damage (24), which can be regulated by Bcl-2 family members (25, 26). Bcl-2 and Bcl-XL operate as critical components in a complex network to integrate information and make ultimate life/death decisions (27). Since the γc-dependent cytokines promote cell survival by upregulating the antiapoptotic factor Bcl-2 (28) and Bcl-XL (29), the expression of Bcl-2 and Bcl-XL in control and X-SCID BCLs was evaluated. In γc-deficient cells, the expression of Bcl-2 and Bcl-XL was greatly decreased as compared with the control (Fig. 1B). These findings indicate that γc is required for cell survival and is dispensable for Fas-induced cell death. Moreover, the evaluation of molecular expression of Bcl-XL in unstimulated cells, through quantitative real-time

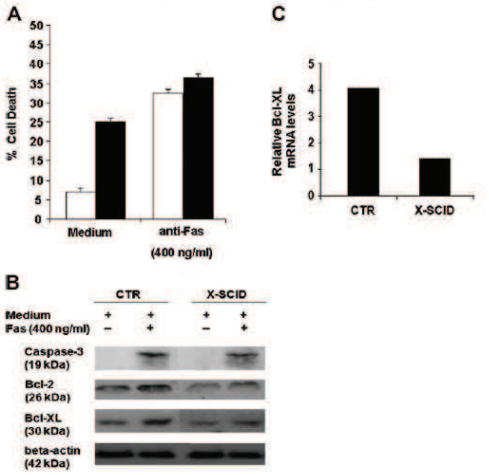

Fig. 1. Deficiency in the expression of γc has effect on cell survival. (A) BCLs were cultured in the absence or presence of 400 ng ml^{-1} anti-Fas for 6 h. The percentage of cell death was evaluated through trypan blue staining. Filled bars indicate BCLs from X-SCID patients; open bars BCLs from healthy donors (CTR). Data are expressed as mean (±SD) of six experiments. (B) BCLs were either cultured in medium alone or stimulated with anti-Fas. After 6 h, whole cell extracts were prepared, caspase-3, Bcl-2 and Bcl-XL expression was determined by western blotting. (C) mRNAs extracted from unstimulated cells were reverse transcribed and analyzed for the expression of Bcl-XL by qRT-PCR. Data were normalized to beta-actin.

PCR, revealed that in the X-SCID cells Bcl-XL mRNA was 35% of the control (Fig. 1C).

Correlation between γc expression and cell proliferation in malignant hematopoietic cell lines

Our previous studies demonstrated that γc exerts a role in cell cycle progression on BCLs in a concentration-dependent manner (11). To explore the potential oncogenic role of γc, we first examined the expression of the molecule in cell lines obtained from hematopoietic tumors, such as Molt-4, Raji, Rj225 and K-562. Synchronization of tumor cells in the G0/G1-phase of the cell cycle was accomplished by 12 h serum starvation. Western blot analysis revealed different γc expression levels among these cell lines. The protein was expressed predominantly in the K-562 and to a lesser extent in the Molt-4, Raji and Rj225 in a decreasing order. In X-SCID BCLs, γc expression was completely undetectable (Fig. 2A). Densitometric analysis is shown in the histogram in Fig. 2(B). The expression of γc was also evaluated in primary leukemic cell lines from three patients with ALL through quantitative real-time PCR. IL-2Rγ mRNA levels were increased in leukemic cells as compared with the control (Fig. 2C).

Moreover, to determine whether the expression levels of γc correlated with the self-sufficient growth in malignant cell lines, we examined the proliferation activity of cells under serum-deficient conditions. This was first evaluated by comparing the CFSE dilution profile, upon trypan blue exclusion

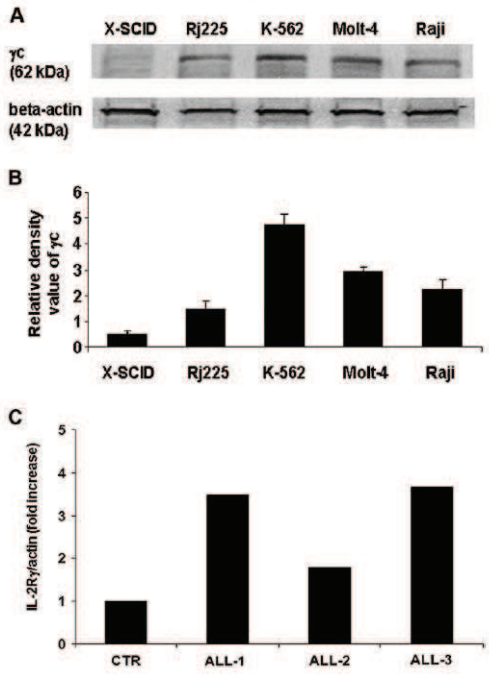

Fig. 2. Different γc expression levels in malignant cell lines. (A) X-SCID BCLs, Burkitt lymphoma cell line (Raji), the chronic myelogenous leukemia cell line (K-562), the human T-ALL cell line (Molt-4) and Raji isogenic derivative (Rj225) were lysed and immunoblotted for γc and beta-actin, as a loading control. (B) Densitometric analysis of the above western blot. ImageJ program was used to generate the data. Data are representative of six distinct experiments. (C) Primary leukemic cell lines, consisting of ALL cells, and control cells were analyzed for the γc expression by qRT-PCR. Histogram shows the relative gene expression as IL-2Rγ/actin fold increase.

assay, of malignant cells. After 5 h of serum-free culture, some variations in the rate of proliferation among the lines were already evident. As shown in Fig. 3(A and B), 20.19% of K-562 retained the dye, indicating a high proliferation rate, compared with 40.65% of Molt-4, 45.2% of Raji and 58.64% of Rj225. No proliferation was observed in X-SCID BCLs (Fig. 3A and B). CFSE experiments, at longer time points, were also performed in order to exclude a delayed entry into the cell cycle in the absence of γc. We found that longer time points were not discriminative as well, since neoplastic cells reach a plateau at 24–72 h of culture (data not shown). Moreover, the proliferation of these cell lines was also assessed by ^{3}H-thymidine incorporation assay after 4 days of culture. K-562 exhibited significantly higher thymidine incorporation (3851 ± 576 cpm) than Molt-4 (2224 ± 167 cpm), Raji (2167 ± 562 cpm) and Rj225 (1534 ± 115 cpm). As expected, there was no proliferation in X-SCID BCLs (Fig. 3C). These data were in keeping with the results of CFSE experiments. A statistically significant relationship between γc expression and spontaneous cell growth was documented in the examined cell lines ($R = 0.98$, $P < 0.05$) (Fig. 3D).

In the light of these findings, we hypothesized that the inhibition of γc expression in hematopoietic malignant cell lines might have a direct effect on proliferation of these cells. siRNA was used to knockdown the molecule in these cell lines. Efficiency and specificity of targeted siRNA sequences were confirmed by western blot analysis on total lysates and quantitative real-time PCR on mRNA. As shown in Fig. 4(A and B), the results of western blot assay revealed that at 96 h following the transfection, cells transduced with siRNA had less γc protein than the correspondent cells transduced with the control negative siRNA. In this representative experiment, γc-silencing reduced the amount of the protein in Rj225, K-562, Molt-4 and Raji by 80, 53, 62 and 32%, respectively. In addition, a decrease of the IL-2Rγ mRNA was observed in all cell lines, revealing a knockdown efficiency of ~85% in this experiment. In X-SCID cells, IL-2Rγ mRNA was undetectable (Fig. 4C). Moreover, as shown in Fig. 4(D), γc knockdown led to a significant decrease of proliferation. In particular, γc-silencing reduced cell proliferation of Rj225 by 40%, K-562 by 58%, Molt-4 by 45% and Raji by 50%, as compared with control siRNA cells (Fig. 4D). Taken together, these data confirm that γc plays a key role in the proliferation of these malignant cell lines.

Effect of γc on molecular mechanisms of cell cycle progression in malignant hematopoietic cell lines

To examine the mechanisms by which γc regulates cell cycle progression, we examined whether different amounts of the molecule were able to influence the transcription of genes selectively involved in cell cycle. Of note, cyclins are the key regulators of cell cycle progression (13). In particular, during the G0 to G1 phase transition, cyclins D1, D2 and D3 are the first molecules to be induced. Cyclin A2 gets activated during the transition from G1 to S phase and B type cyclins are detected during G2 exit and mitosis phase (30). In several kinds of malignant tumors, cyclins are overexpressed (31). Therefore, in all the above described cell lines, quantitative real-time analysis was performed to examine the effect of γc on the expression of cyclin A2, B1, D1, D2, D3 genes. As shown in Fig. 5(A), K-562 exhibited an up-regulation of D-type cyclins to a similar extent of Molt-4, while these genes were not expressed in Raji and X-SCID BCLs. In Rj225, only the cyclin D3 mRNA was up-regulated. Interestingly, we found that cyclins A2 and B1 were undetectable in X-SCID BCLs and reached a maximal expression in K-562 (Fig. 5B). In Molt-4, Rj225 and Raji, the levels of the cyclins A2 and B1 were lower than in K-562 (Fig. 5B). A direct correlation between cyclins A2 and B1 and γc expression was found ($P < 0.05$) (Fig. 5C and D). These data are consistent with the direct correlation previously shown between functional and molecular data of γc amount.

Discussion

Our results first indicate that in the absence of γc expression, BCLs die at a higher extent than control cells. This phenomenon is not related to abnormalities of regulatory

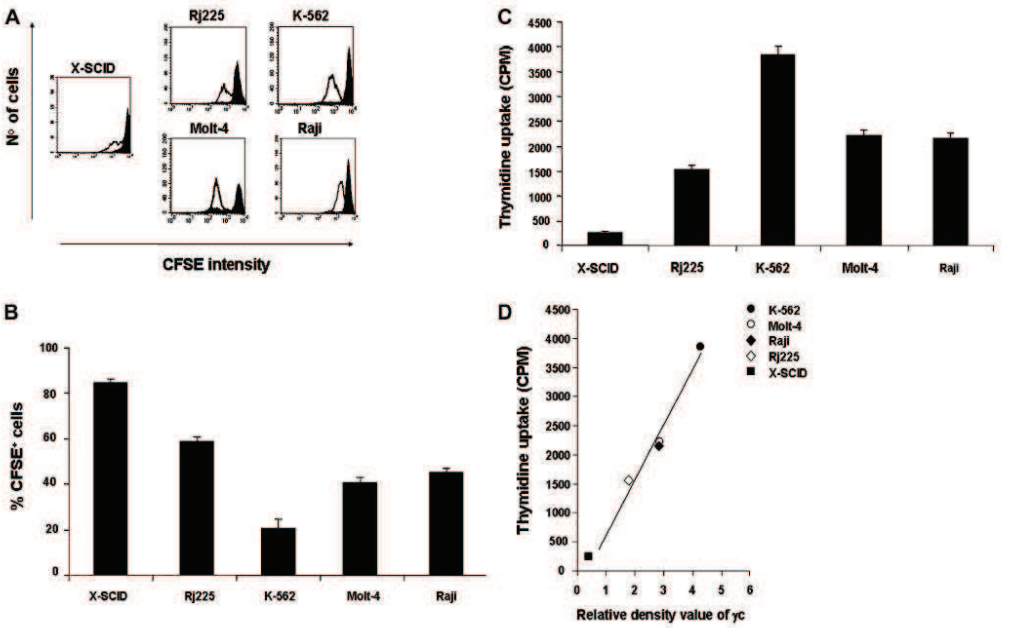

Fig. 3. Relationship between γc expression and spontaneous cell growth in malignant cell lines. (A) X-SCID BCLs, Raji, K-562, Molt-4 and Rj225 were cultured in the absence of serum and stained with 1.7 μM CFSE. After 6 h of culture, cells were analyzed by flow cytometry. Histograms show CFSE profiles 6 h following the start of culture. Solid black peaks represent the start of the culture. (B) Percentages of CFSE positive cells were obtained in the indicated cell lines by flow cytometry. Graphical representation of the mean (±SD) of percentage of CFSE positive cells for the three experiments conducted. (C) After starvation, X-SCID BCLs, Rj225, K-562, Molt-4 and Raji were cultured in serum-free medium for 4 days and pulsed with 0.5 μCi ^{3}H-thymidine for 8 h. Data represent mean (±SD) of six experiments. (D) Correlation between thymidine incorporation and γc expression in malignant cells. A positive correlation was demonstrated by the Pearson correlation coefficient ($R = 0.98$, $P < 0.05$).

mechanisms of apoptosis, in that, in non-stimulated cells, the absence of γc does not induce an increase of the activated form of caspase-3 that represents the central executioner molecule in the development of programmed cell death process. Hallmark of non-caspase-mediated cell death is the mitochondrial damage (24), which can be regulated by Bcl-2 family members (25). The γc-dependent cytokines promote cell survival by up-regulating the antiapoptotic Bcl-2 and Bcl-XL factors. In keeping with this, we found a decreased expression of Bcl-2 and Bcl-XL in γc-deficient cells. Of note, this effect was related to the lack of γc *per se* and not a secondary effect of unfunctional γc-dependent cytokines IL-2 or IL-4, in that neutralization of these cytokines did not modify cell viability of normal cells. As for the mechanism of non-caspase-mediated apoptosis, there is evidence indicating that autophagy may play an active role in cell death under unfavorable settings, such as nutrient or growth factors deprivation (27). However, whether γc is implicated in the autophagy process still needs to be addressed.

In spite of a wide number of studies on the molecular interactions and functions of γc, the precise role of this molecule in cell biology is still far from being clear. It has recently been documented a direct relationship between the amount of γc expression and the proliferative capability of control lymphoblastoid cells (11), implying a direct involvement of γc in self-sufficient growth in a concentration-dependent manner. Moreover, it was previously reported that *IL2RG* cooperates with *LMO2* in inducing hematopoietic tumors by studies of insertional mutagenesis in mice (32), thus giving a potential explanation to lymphoproliferative disorders occurring during gene therapy trials for X-SCID (9, 10). It is noteworthy that, differently from X-SCID, no clonal lymphoproliferation has been reported, to date, in patients receiving gene therapy for ADA deficiency (33), despite the observation of a similar frequency of integration sites near *LMO2* and other proto-oncogenes (34). Furthermore, a recent study, based on an experimental model of gene transfer in γc−/− mice, documented that γc overexpression could exert oncogenic properties by itself (35).

In this study, we observed that the amount of γc protein expression in several malignant cell lines directly correlates with spontaneous cell growth. We, also, found that the knockdown of γc molecule through siRNA is able to decrease the cell proliferation rate in these malignancies, thus confirming a direct involvement of the molecule as a key player in cell cycle progression.

The cancer is a multistep process that requires mutations of multiple molecules implicated in the biochemical signaling

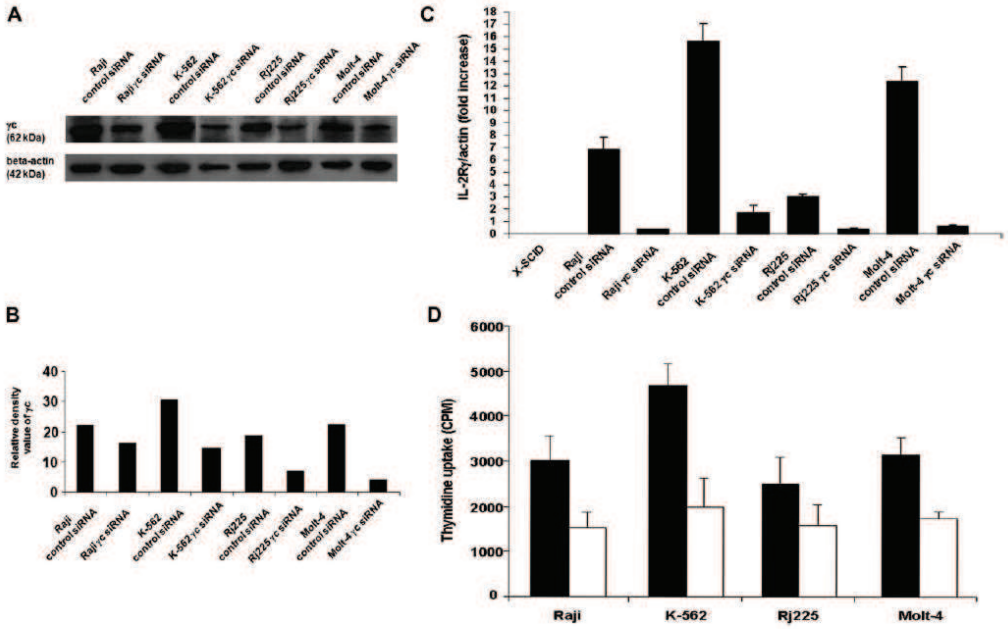

Fig. 4. Effect of γc siRNA transfection on the expression level of protein and cell proliferation of malignant cell lines. (A) Western blot analysis of γc and beta-actin protein expression in Rj225, K-562, Molt-4 and Raji transfected with γc siRNA or transfected with negative control siRNA after 96 h of transfection. (B) Densitometric analysis of the above western blot. ImageJ program was used to generate the data. Data were equalized for the background. Results are representative of five distinct experiments. (C) IL-2Rγ mRNA transcript evaluated by quantitative real-time PCR. Relative mRNA expression was determined using beta-actin control. (D) The proliferation of Rj225, K-562, Molt-4 and Raji transfected with γc siRNA or transfected with negative control siRNA was evaluated through ^{3}H-thymidine incorporation assay. Data represent mean (±SD) of five distinct experiments.

events of cell proliferation, thus providing growth advantage to the malignant cell (36). It is known that constitutive activation of JAK3 has been observed in a spectrum of lymphoid malignancies (37). In particular, JAK overexpression can be considered as one of the main biologic events leading to the constitutive activation of the JAK-STAT pathway that contributes to oncogenesis (38). It has been demonstrated that the amount of constitutively activated JAK3 parallels the extent of γc expression (11). Moreover, STAT molecules and, in particular the nearly identical STAT5 A and B (39), have been demonstrated to directly participate in tumor development and progression. STAT5 participates in oncogenesis through up-regulation of genes encoding cell cycle regulators, such as cyclins (40). Alterations in cell cycle machinery, mainly in the regulation of G1/S phase, are known to be associated to the development of solid tumors as well as hematological malignant diseases (31). In this context, a direct involvement of cyclins in malignant cell growth has been well documented and a correlation between the extent of cyclins expression/activation and the rate of proliferation has been found. Namely, cyclins A2 and B1 have been implicated in the pathogenesis of cancer and are overexpressed in several tumors (41, 42). Evidence indicates that these cyclins are key components of the cell-cycle machinery (43) and, in particular, cyclin A is expressed at high levels in hematopoietic stem cells and is essential for their proliferation (44). In our study, we observed that the expression of A2 and B1 cyclins strongly paralleled the proliferative capability of malignant cell lines. Interestingly, a positive correlation between the amount of γc and the expression of cyclins A2 and B1 was also found. Taken together, these data indicate that the higher is the rate proliferation of a certain cell line the higher is the expression of both γc and cyclins A2 and B1, thus confirming their involvement in the process in a concentration-dependent fashion.

We also found an increased expression of all D-type cyclins in those cell lines that proliferated mostly, K-562 and Molt-4, whereas they were not expressed in the other cell lines, but D3 found in Rj225. D-type cyclins are strongly expressed in many malignancies. Overexpression of cyclin D1 protein was documented in many forms of cancer, including breast cancer (45), while overexpression of cyclin D2 was noted in a wide range of B-cell malignancies, such as B-cell chronic lymphocytic leukemia (46). Like the other D cyclins, cyclin D3 is rearranged and the protein is overexpressed in several human lymphoid malignancies. It was documented that knockdown of cyclin D3 inhibits the proliferation of ALL cells (47). However, while A- and B-type cyclins seem to be vital and necessary components of cell cycle progression (44), D-type cyclins may be dispensable

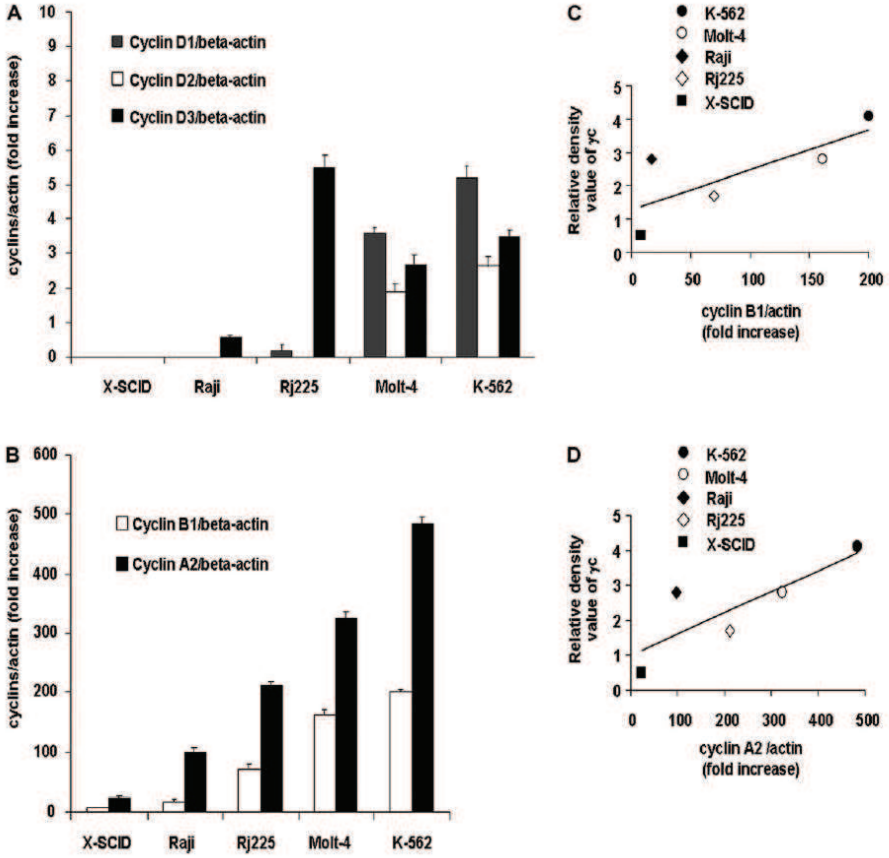

Fig. 5. Cyclins expression is up-regulated in malignant cell lines. (A–B) RNAs extracted and reverse transcribed were analyzed for the expression of D1, D2, D3 and A2, B1 cyclins by qRT-PCR. Histograms show the relative gene expression as cyclin/actin fold increase. Relative expression of cyclins were calculated for each cell line after normalizing against beta-actin. (C) Correlation between γc protein amount, expressed as relative density, and fold increase cyclin B1/actin expression. (D) Correlation between γc protein amount, expressed as relative density, and fold increase cyclin A2/actin expression.

for proliferation under certain circumstances, in that different cell types are sensitive to cyclin D knockdown at a different extent (48). This would suggest that they regulate cell cycle in a cell type-specific manner and that there are alternative mechanisms allowing cell cycle progression in a cyclin D-independent fashion (48). Anyway, a critical role for oncogenic transformation of D-type cyclins is a well-established feature.

Our data indicate that γc is strongly implicated in cell cycle progression of hematopoietic malignancies in a similar fashion to the role played in control lymphoblastoid cells, as previously shown. This biologic effect is strictly dependent on the expression level of the molecule and can be abolished by gene knockdown. Of note, a direct correlation between the amount of γc expression and the proliferative capability of the malignant cell lines and the regulatory elements of cell cycle progression, A and B cyclins, was found. Moreover, we documented that the IL-2Rγ mRNA was also highly expressed in primary leukemic cells, thus confirming a direct involvement of the γc in tumor growth. Our data could provide the basis to develop in the near future new therapeutic strategies targeting this molecule in cancer therapy. Moreover, this information may also help understand undesired side effects of gene therapy trials.

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References

1 Leonard, W. J. 1996. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. *Annu. Rev. Med.* 47:229.
2 Notarangelo, L. D., Giliani, S., Mazza, C. *et al.* 2000. Of genes and phenotypes: the immunological and molecular spectrum of combined immune deficiency. Defects of the gamma(c)-JAK3 signaling pathway as a model. *Immunol. Rev.* 178:39.
3 Rochman, Y., Spolski, R. and Leonard, W. J. 2009. New insights into the regulation of T cells by γ_c family cytokines. *Nature Rev. Immunol.* 9:480.
4 Yao, Z., Cui, Y., Watford, W. T. *et al.* 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl Acad. Sci. USA* 103:1000.
5 Moriggl, R., Topham, D. J., Teglund, S. *et al.* 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 10:249.
6 Lord, J. D., McIntosh, B. C., Greenberg, P. D. *et al.* 2000. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *J. Immunol.* 164:2533.
7 Lockyer, H. M., Tran, E. and Nelson, B. H. 2007. STAT5 is essential for Akt/p.70S6 kinase activity during IL-2-induced lymphocyte proliferation. *J. Immunol.* 179:5301.
8 Barata, J. T., Keenan, T. D., Silva, A. *et al.* 2004. Common γ chain-signaling cytokines promote proliferation of T-cell acute lymphoblastic leukemia. *Haematologica* 89:1459.
9 Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P. *et al.* 2008. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* 118:3132.
10 Howe, S. J., Mansour, M. R., Schwarzwaelder, K. *et al.* 2008. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J. Clin. Invest.* 118:3143.
11 Amorosi, S., Russo, I., Amodio, G. *et al.* 2009. The cellular amount of the common γ-chain influences spontaneous or induced cell proliferation. *J. Immunol.* 182:3304.
12 Maddika, S., Ande, S. R., Panigrahi, S. *et al.* 2007. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. *Drug Resist. Updat.* 10:13.
13 Sherr, C. J. and Roberts, J. M. 2004. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev.* 18:2699.
14 Lindemann, M. J., Benczik, M. and Gaffen, S. L. 2003. Anti-apoptotic signalling by the interleukin-2 receptor reveals a function for cytoplasmic tyrosine residues within the common gamma (gamma c) receptor subunit. *J. Biol. Chem.* 278:10239.
15 Martino, A., Holmes, J. H. IV, Lord, J. D. *et al.* 2001. Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-21. *J. Immunol.* 166:1723.
16 Moon, J. J., Rubio, E. D., Martino, A. *et al.* 2004. A permissive role for phosphatidylinositol 3-kinase in the Stat5-mediated expression of cyclin D2 by the interleukin-2 receptor. *J. Biol. Chem.* 279:5520.
17 Engedal, N., Gjevik, T., Blomhoff, R. *et al.* 2006. All-trans retinoic acid stimulates IL-2-mediated proliferation of human T lymphocytes: early induction of cyclin D3. *J. Immunol.* 177:2851.
18 Zinkel, S., Gross, A. and Yang, E. 2006. BCL2 family in DNA damage and cell cycle control. *Cell Death Differ.* 13:1351.
19 Cooper, A. B., Sawai, C. M., Sicinska, E. *et al.* 2006. A unique function for cyclin D3 in early B cell development. *Nat. Immunol.* 7:489.
20 Pignata, C., Troncone, R., Monaco, G. *et al.* 1985. Impaired suppressor activity in children affected by coeliac disease. *Gut* 26:285.
21 Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using Real-Time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402.
22 DeFranco, S., Bonissoni, S., Cerutti, F. *et al.* 2001. Defective function of Fas in patients with type 1 diabetes associated with other autoimmune diseases. *Diabetes* 50:483.
23 Lamkanfi, M., Festjens, N., Declercq, W. *et al.* 2007. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ.* 14:44.
24 Tait, S. W. G. and Green, D. R. 2008. Caspase independent cell death: leaving the set without the final cut. *Oncogene* 27:6452.
25 Haraguchi, B. M., Torii, S., Matsuzawa, S. *et al.* 2000. Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. *J. Exp. Med.* 191:1709.
26 Hua, F., Cornejo, M. G., Cardone, M. H. *et al.* 2005. Effects of Bcl-2 levels on Fas signaling-induced Caspase-3 activation: molecular genetic tests of computational model predictions. *J. Immunol.* 175:985.
27 Zhou, F., Yang, Y. and Xing, D. 2011. Bcl-2 and Bcl-xL play important role in the crosstalk between autophagy and apoptosis. *FEBS J.* 278:403.
28 von Freeden-Jeffry, U., Solvason, N., Howard, M. *et al.* 1997. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity* 7:147.
29 Akbar, A., Borthwick, N. J., Wickremasinghe, R. G. *et al.* 1996. Interleukin-2 receptor common γ-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *Eur. J. Immunol.* 26:294.
30 Budirahardja, Y. and Gonczy, P. 2009. Coupling the cell cycle to development. *Development* 136:2861.
31 Ekberg, J., Holm, C., Jalili, S. *et al.* 2005. Expression of cyclin A1 and cell cycle proteins in hematopoietic cells and acute myeloid leukemia and links to patient outcome. *Eur. J. Haematol.* 75:106.
32 Davé, U. P., Akagi, K., Tripathi, R. *et al.* 2009. Murine leukemias with retroviral insertions at Lmo2 are predictive of the leukemias induced in SCID-X1 patients following retroviral gene therapy. *PLoS Genet.* 5:e1000491.
33 Aiuti, A., Cassani, B., Andolfi, G. *et al.* 2007. Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy. *J. Clin. Invest.* 117:2233.
34 Deichmann, A., Hacein-Bey-Abina, S., Schmidt, M. *et al.* 2007. Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J. Clin. Invest.* 117:2225.
35 Woods, N. B., Bottero, V., Schmidt, M. *et al.* 2006. Woods et al. reply. *Nature* 443:E6.
36 Du, Y., Spence, S. E., Jenkins, N. A. *et al.* 2005. Cooperating cancer-gene identification through oncogenic-retrovirus-induced insertional mutagenesis. *Blood* 106:2498.
37 Cornejo, M. G., Kharas, M. G., Werneck, M. B. *et al.* 2009. Constitutive JAK3 activation induces lymphoproliferative syndromes in murine bone marrow transplantation models. *Blood* 113:2746.
38 Knoops, L., Hornakova, T., Royer, Y. *et al.* 2008. JAK kinases overexpression promotes in vitro cell transformation. *Oncogene* 27:1511.
39 Ambrosio, R., Fimiani, G., Monfregola, J. *et al.* 2002. The structure of human STAT5A and B genes reveals two regions of nearly identical sequence and an alternative tissue specific STAT5B promoter. *Gene* 285:311.
40 Harir, N., Pecquet, C., Kerenyi, M. *et al.* 2007. Constitutive activation of Stat5 promotes its cytoplasmic localization and association with PI3-kinase in myeloid leukemias. *Blood* 109:1678.
41 Yam, C. H., Fungand, T. K. and Poon, R. Y. C. 2002. Cyclin A in cell cycle control and cancer. *Cell. Mol. Life Sci.* 59:1317.
42 Yuan, J., Yan, R., Kramer, A. *et al.* 2004. Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. *Oncogene* 23:5843.
43 Hochegger, H., Takeda, S. and Hunt, T. 2008. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nature* 9:910.

44 Kalaszczynska, I., Geng, Y., Lino, T. *et al.* 2009. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. *Cell* 138:352.
45 Landis, M. W., Pawlyk, B. S., Li, T. *et al.* 2006. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. *Cancer Cell* 9:13.
46 Decker, T., Schneller, F., Hipp, S. *et al.* 2002. Cell cycle progression of chronic lymphocytic leukemia cells is controlled by cyclin D2, cyclin D3, cyclin-dependent kinase (cdk) 4 and the cdk inhibitor p.27. *Leukemia* 16:327.
47 Sicinska, E., Aifantis, I., Le Cam, L. *et al.* 2003. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4:451.
48 Kozar, K., Ciemerych, M. A., Rebel, V. I. *et al.* 2004. Mouse development and cell proliferation in the absence of D-cyclins. *Cell* 118:477.

2.3. γc over-expression in B-precursor acute lymphoblastic leukemia primary cell lines from pediatric patients: implication in leukemia cell cycle progression and survival.

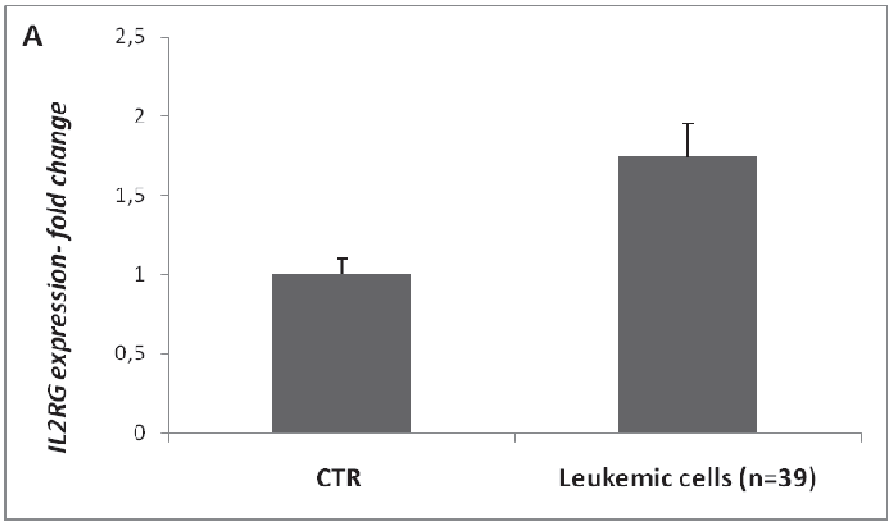
In our previous studies, we observed that γc exerts a role in either spontaneous or GH-induced cell cycle progression, depending on the amount of the protein expression (174). Moreover, we documented an over-expression of γc protein in continuous malignant hematopoietic cell lines, and a direct correlation between its expression and spontaneous cell growth. In addition, an increased expression of all D-type cyclins and a direct correlation between the amount of γc and cyclins A2 and B1 expression was noted, thus implying a critical regulatory role of γc on cell proliferation of continuous malignant hematopoietic cells (175).

Of note, it is well known that γc-receptors play a role in T-cell development and function, Under physiological circumstances, and in T-cell Acute Lymphoblastic Leukemia (T-ALL) leukemogenesis (176, 177).

In order to evaluate whether γc-receptors are involved in biology of specific type of leukemia, and not only in T-ALL, analyzed the γc expression profile and γc-signaling in different types of leukemia. We evaluated the γc expression in γc primary cells of Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), B-precursor Acute Lymphoblastic Leukemia (B-pre ALL), and T-ALL. We also investigated the potential mechanisms by which γc exerts its role in leukemia development and progression.

Particularly, we evaluated the expression levels of *IL-2Rγ* mRNA in primary cells derived from bone marrow of 39 newly diagnosed patients with CML, AML, B-pre ALL and T-ALL, and control, by performing quantitative Real-Time PCR. We selected

only newly diagnosed patients to avoid interpretation bias due to use of chemotherapeutic agents. As show in **Figure 7A**, *IL-2Rγ* mRNA expression was increased in leukemic cells (mean ± SE = 1,74 ± 0,21) as compared with the control. In addition, *IL-2Rγ* was predominantly expressed in lymphoblastic leukemia cells (mean ± SE = 2,62 ± 0,4) (**Figure 7B**).



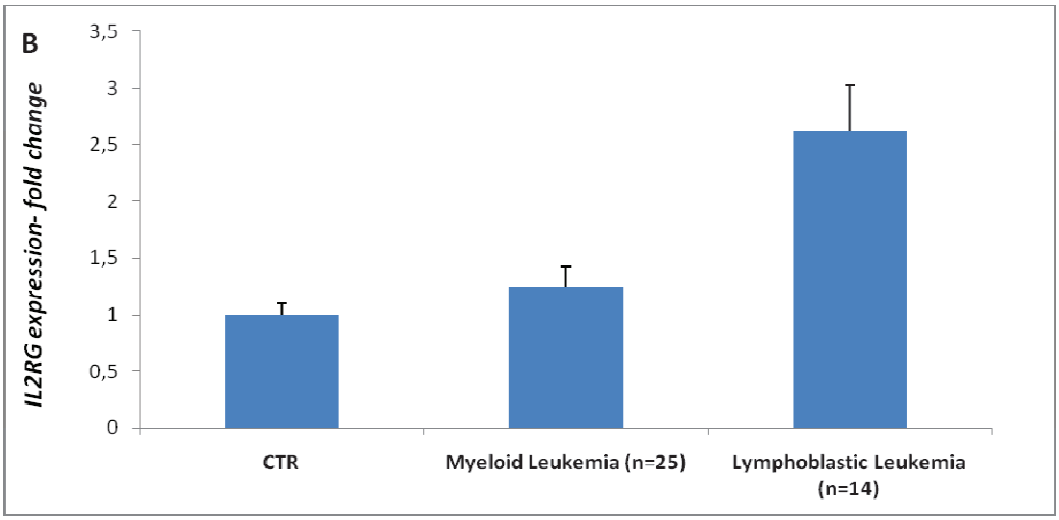


Figure 7: *IL2Rγ* mRNA expression was increased in leukemia cells. Total RNA from leukemic cells and controls was extracted and reverse transcribed. The expression levels of *IL2GR* were analyzed by qRT-PCR in leukemia cells from AML, CML, ALL patients and controls. (B) *IL2GR* mRNA amount was mainly increased in ALL cells. The relative gene expression is a ratio *IL2Rγ*/ β-actin fold change. Each histogram represents the mean ± SE.

γc-signaling cytokines play a central role in T-cell development and in the mitogenic potential of primary T-ALL cells (176), suggesting the implication of γc-signaling in this leukemia subtype. Differently from what expected, we found a higher expression of *IL-2Rγ* mRNA in B-pre ALL (mean ± SE = 2,92 ± 0.5) than in T-ALL cells (mean ± SE = 1,80 ± 0,3) (**Figure 8**). These data suggest a still unappreciated role of γc in B-pre ALL cells.

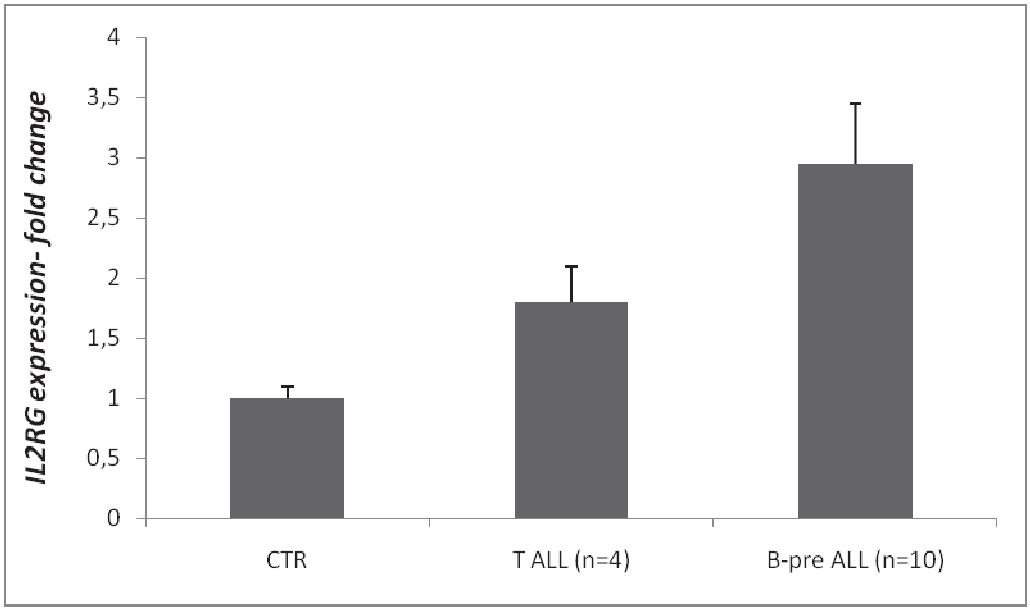


Figure 8: *IL2Rγ* mRNA expression was higher expression in B-pre ALL than in T-ALL cells. The expression levels of *IL2GR* were analyzed by qRT-PCR. The relative gene expression is a ratio *IL2Rγ*/ β-actin fold change. Each histogram represents the mean ± SE.

The master regulator proteins of cell cycle progression are the cyclin-cdk complexes, an evolutionarily conserved family of proline-dependent serine/threonine kinases (178).

In ALL abnormal expression and function of cell cycle proteins has already been documented (179), and, in particular, in B-cell malignancies of D-type cyclins (180) under control of PI3-AKT signaling pathways, the latter being, in turn, regulated by γc. In keeping with this, we investigated the potential regulation of cell cycle progression

process promoted by γc in B-pre ALL cells, by evaluating expression pattern of D-type cyclins through Real-Time PCR.

The results showed that D-type cyclins were expressed at higher extent in B-pre ALL cells than controls, as shown in figure 3. In particular, all the three cyclins were over-expressed in the B-pre ALL (D1 cyclin: mean ± SE = 21.90 ± 2.8; D2 cyclin: mean ± SE = 5.18 ± 0.80); D3 cyclin mean ± SE = 3.22 ± 0.61) (**Figure 9**).

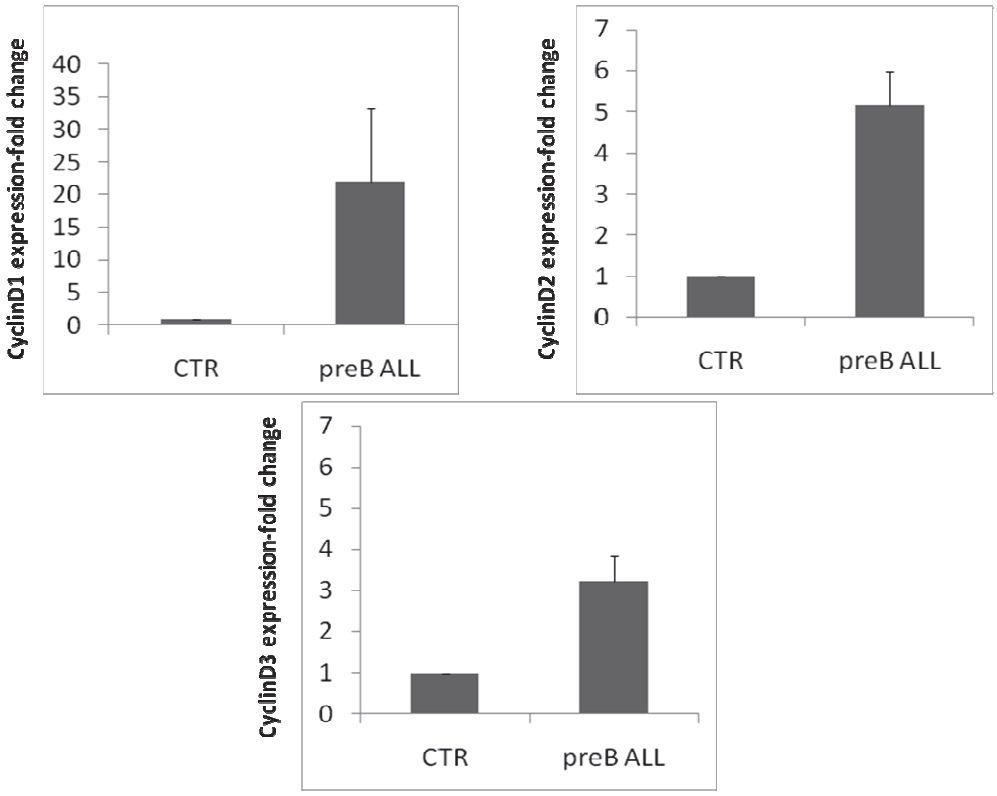


Figure 9: *D1*, *D2* and *D3* cyclins expression levels were increased in B-pre ALL cells. Analysis of D-type cyclins expression using qRT-PCR revealed up-regulation of all D cyclins. The mRNA levels were expressed relative to β-actin expression levels. Each histogram represents the mean ± SE.

We next correlated the expression levels of cyclins D1, D2, D3 with γc. As shown in Figure 4, a direct correlation between γc and cyclins D2 (R = 0.82) and D3 (R = 0.76) expression was found, while no correlation between γc expression and cyclin D1 was observed (**Figure 10**).

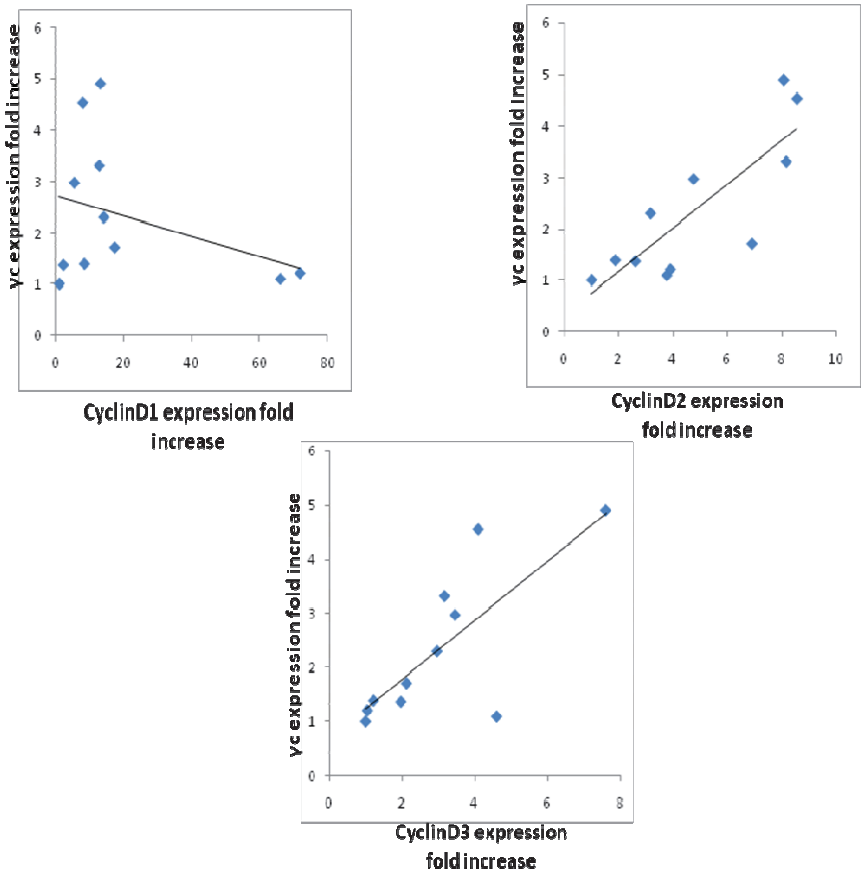


Figure 10: Relationship between *IL2R*γ and cyclins D1, D2 , D3 expression levels. A direct positive correlation between *IL2R*γ and cyclins D2 and D3 was found, as demonstrate by value of Pearson correlation coefficient (cyclin D2: $P = 0.82$; cyclin D3: $P = 0.76$). Any correlation between *IL2R*γ and cyclins D1 was found.

Cancer development and progression mainly relies on the ability of tumor cells to escape apoptosis more than their normal counterparts (181). Apoptosis is regulated by BCL-2 family members, including BCL-2, BCL-XL, BAX, BAD (182). In keeping with this, an abnormal expression or function of BCL-2 family proteins has been well documented in human cancer, leading to programmed cell death evasion, and chemotherapy resistance (183). Noteworthy, γc is also implicated in regulating genes involved in this biological process through the activation of PI3-K/AKT signaling pathway (184).

To investigate the mechanisms by which γc promotes survival in B-pre ALL leukemic cells, we evaluated the expression level of the anti-apoptotic BCL-XL by quantitative real-time PCR. We observed that in primary B-pre ALL leukemic cells, BCL-XL mRNA was 57% than control (mean ± SE = 0.57 ± 0.12) (**Figure 11**), thus suggesting the presence of alternative mechanisms of the pro-survival effect of γc.

Recent evidence suggests that autophagy favours cell survival in response to multiple stresses, including hypoxia, nutrient deprivation, and other events occurring in the primary tumor microenvironment (185, 186, 187). BCL-2 family proteins are also involved in autophagy. Indeed, they are able, through their BH3-like domain, to bind and inhibit the activation of BECLIN-1, an important mediator of autophagy,

Therefore, by using quantitative Real-Time PCR, we observed that the expression level of *BECLIN-1* mRNA was increased (mean ± SE = 4.73 ± 0.71) in B-pre ALL leukemic cells as compared with the controls (**Figure 11**), suggesting a putative role of γc in promoting cell survival in B-pre ALL *via* autophagy.

Figure 11

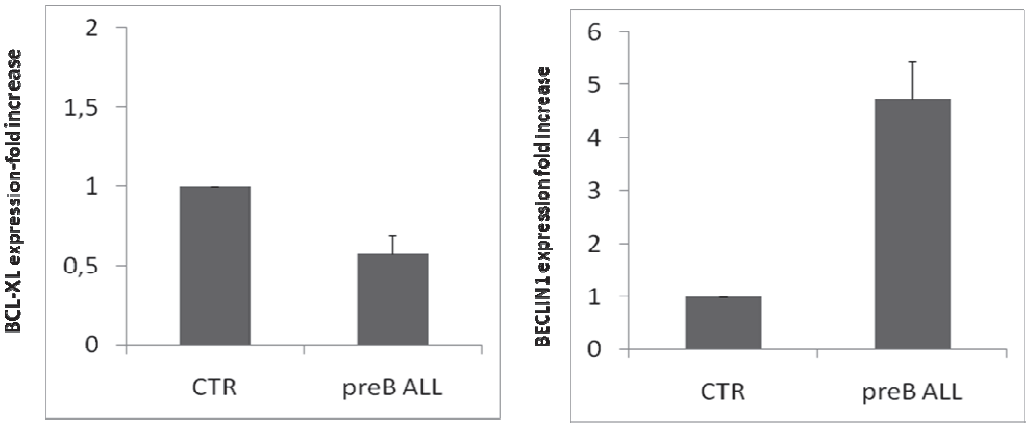


Figure 11: γc promote leukemia cell survival *via* autophagy through the up-regulation of BECLIN-1. BCL-XL and BECLIN-1 mRNA expression was analyzed using qRT-PCR. *BCL-XL* mRNA was 57% of controls. *BECLIN-1* expression was greatly increased. The mRNA levels were expressed relative to β-actin expression levels. Each histogram represents the mean ± SE.

Western Blot analysis on total lysates obtained from four B-pre ALL patients, confirmed the overexpression of BECLIN-1, as compared with normal control (**Figure 12**).

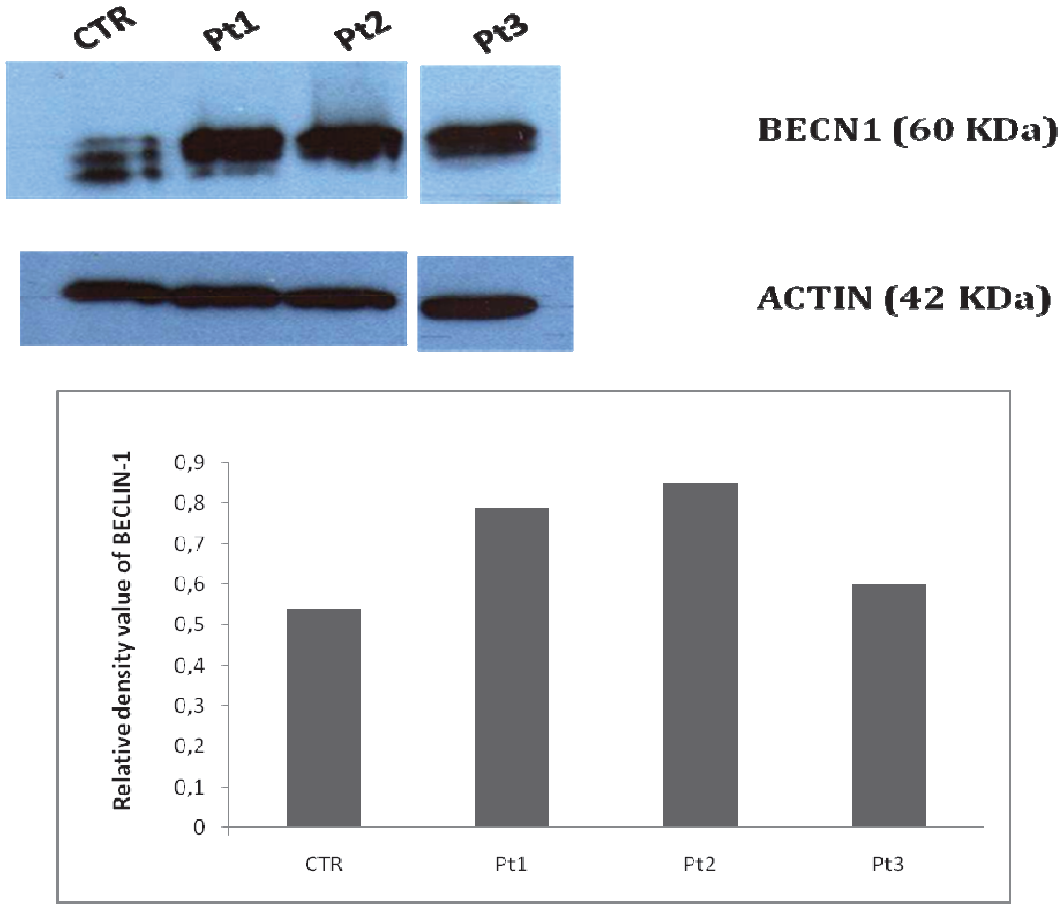


Figure 12: Western Blot analysis revealed over-expression of BECLIN-1 in B-pre ALL, as compared with control. Whole cell extracts were prepared from B-pre ALL cells and, BECLIN-1 expression was determined by western blotting. Densitometric analysis of the above western blot was performed by using ImageJ program.

The regulatory role of a number of cytokines, such as interleukins and colony-stimulating factors, on the survival, the growth, the differentiation and the apoptosis in leukemic cells, both *in vitro* and *in vivo*, has been documented (188). Of note, by using of knock-out strategy or through the neutralization of cytokines γc-receptors, the

clinical implication of γc-dependent signaling has been shown, suggesting that the increasing or decreasing of this cascade could be helpful in cancer treatment (131).

Our results showed that *IL2RG* was over-expressed in leukemia cells, as compared with controls. In particular, we observed that *IL2RG* mRNA was mainly increased in ALL cells. Moreover, we found a predominant up-regulation of *IL2RG* expression levels in B-pre ALL cells, differently from what expect, because it is well documented the role of γc-signaling cascade in physiological T-cell development and evidence of involvement of γc in biology of T-ALL are available (176). Our data demonstrate, for the first time, a selective over-expression of *IL2RG* mRNA in B-pre ALL cells, suggesting a specific role of molecule in this leukemia type.

Tumor cells exhibit the aberrant ability to proliferative without control due to mutations affecting genes involved in cell cycle progression, a tightly regulated process (146). The passage through the early G1 and late G1 phase of cell cyle represents a critic check-point that irreversibly directs the cells to undergo one cell division (189). D-type cyclins are important regulators of this process (190) and their abnormal activation or overexpression contribute to pathogenesis of lymphoproliferation (191). The overexpresssion of D-type cyclins in B-cell malignancies has been reported (192). In addition, in ALL alterations in *cyclins D1*, *D2* and *D3* have been documented (193, 194, 195). Eventually, it has been shown that activation of *cyclin D-type* expression is dependent upon induction of PI 3-kinase/Akt kinase, an important signaling pathways downstream γc. In our study, we observed that *cyclins D1*, *D2*, *D3* mRNA expression levels were up-regulated in B-pre ALL, as compared with controls. In particular, *cyclins D1* mRNA was mainly increased. Interestingly, a direct positive correlation between the amount of γc and the expression of *cyclins D2* and *D3* was also found. These data

demonstrate not yet documented role of γc in promoting cell cycle progression of B-pre ALL cells through up-regulation of *cyclins D2* and *D3* expression.

The impairment of apoptosis is a critical step in cancer development (148), and BCL-2 family proteins play a pivotal role in promoting tumor cell survival (150). It has been documented that BCL-XL protein is expressed at high levels in childhood ALL (196, 197). Of note, the γc signaling pathway leads to selective induction of anti-apoptotic (Bcl-2 and Bcl-xL) gene expression (162). Differently from what expect, we found a reduced mRNA BCL-XL level, as compared with controls. This finding suggested that B-pre ALL cells, over-expressing γc, are able to escape cell death through alternative mechanisms.

The high proliferation rate of cancer cells requires continuous source of energy and nutrient, but the tumor microenvironment is not able to supply these essential need for cancer cell survival (198). Under these conditions, the cancer cells undergo a shortage of nutrients, thus activating alternative metabolic processes to escape this stress and maintain their survival. Of note, several studies reported on a critical role of autophagy in protecting cells against a shortage of nutrients, through removal and recycling of damaged molecules and organelles, but the exact molecular and biochemical mechanism by which cancer cells obtain energy sources in condition of lack of external nutrients remains to be elucidate (199, 200). However, evidence indicates that the predominant role of autophagy in cancer cells is to confer stress tolerance, which allows tumor cell survival (201).

As for speculated wheatear autophagy activation occurred in B-pre ALL cells, we evaluated BECLIN-1 mRNA levels and protein amount in B-pre ALL cells and we

found a prominent up-regulation of BECN-1 mRNA levels and protein expression in B-pre ALL cells, as compared with controls, which suggested autophagy activation..

Beclin-1, a part of a class III phosphatidylinositol-3-kinase (PI(3)K) complex, participates in autophagosome nucleation (202). Beclin-1 is activated by a number of proteins (AMBRA1, UVRAG and Bif-1) (203, 204, 205), promoting the activation of the PI(3)K protein (Vps34) and the formation of autophagosomes, while its autophagy-promoting ability is suppressed by antiapoptotic Bcl-2 family members through direct binding of BH3-domain-only.

Of note, the decreased expression of BCL-XL in our leukemic cells, and thus the absence of inhibitory effect exerted by this protein on BECN-1, consistent with up-regulation of BECN-1 expression, supported our hypothesis of autophagy activation.

In conclusion, we documented for the first time a selective up-regulation of *IL2RG* expression in B-pre ALL cells. Moreover, we demonstrated a direct correlation between mRNA levels of *IL2RG* and *cyclins D2* and *D3*, thus implying a role of γc in promoting cell cycle progression in B-pre ALL cells by transcriptional regulation of these genes. Eventually, we reported on the regulation of B-pre ALL cells survival through the activation of autophagy, as suggested by BECN-1 increased expression, even though additional evidence of occurrence of this process as well as the characterization of the role of γc in autophagic cell survival have to be provided.

The identification of critical role of γc in pathophysiology should contribute to develop more innovative and effective therapies, aimed to inhibit proliferative and pro-survival effects activated by γc-receptors.

2.4. γc transducing element as a functional link between endocrine and immune system.

Immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion (139). Several evidences demonstrate the existence of a previously unappreciated relationship between distinct elements, such as GH-R and γc and their signaling pathways. Crosstalk between receptor signaling systems is now emerging as an important and exciting area of signaling research. The impairment of various GH-induced events in γc deficiencies suggests a potential interaction between GH-R pathways and γc, indicating a further functional link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. The existence of shared molecular mechanisms of transduction between immune and endocrine systems is well documented in several autoimmune disorders characterized by both endocrine and immune features (206, 207, 208). GH-R pathways and γc interaction leads to the activation and intranuclear translocation of STAT5b protein. The signals mediated by STATs generally play a central role in the control of important cellular events such as cell proliferation, differentiation and apoptosis (71), even though the overall role of the STAT molecules in GH-R signal transduction has not been fully elucidated. The overall signal transduction properties of GH-R in X-SCID patients and control BCLs following GH stimulation, have been recently elucidated. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in γc negative patients cell lines in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of XSCID cells with WT γc gene corrected the functional and biochemical abnormalities resulting in an

appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γc in GH-R signaling. Of note, a colocalization of GH-R and γc has been documented. Even though a physical interaction between the two proteins may be possible, any physical association has not yet been documented (136). Currently, studies are ongoing to identify potential physical interactions between the two proteins. Moreover, evidence suggests that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression. Whether the participation of γc to the GH-R confers some additional properties to the receptor in hematopoietic cell differentiation and functioning remains to be elucidated.

The evidence of this relationship have been published as *Review* on *Current Signal Transduction Therapy*, for the manuscript see below.

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Current Signal Transduction Therapy, 2013, *8*, 67-73

Networking Between γc and GH-R Signaling in the Control of Cell Growth

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Abstract: The family of type I cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, shares common transducing element the common cytokine receptor γc. The receptors containing γc exert prominent mitogenic effects and play an important role in several immunological functions and in supporting cell survival. The γc-dependent cytokine receptors use the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway to mediate gene activation or repression. The Growth Hormone (GH) is a peptide of 191 amino acids and 22 kDa molecular weight, produced by the adenohypophysis, that regulates many important functions, as control of cellular metabolism, immune functions, fertility and somatic growth. Of note, the existence of a previously unappreciated functional interaction between γc and Growth Hormone receptor (GH-R) has been recently documented. The impairment of various GH-induced events in patients affected with severe combined immunodeficiencies due to γc defects suggests a potential functional interaction between GH-R pathways and γc, indicating a further link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. GH-R pathways and γc interaction leads to the activation and intranuclear translocation of STAT5b protein. Moreover, evidence suggests that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on the amount of γc expression. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a proper functionality of γc-dependent cytokines on the whole seems to play prominent roles, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system.

Keywords: Cell growth, γc, GH, GH-R, JAKs, STATs.

INTRODUCTION

The cytokines and growth factors are important regulator elements. They transduce signals to the nucleus though specific cell-surface receptors and the process ultimately results in the activation of transcription factors [1]. Cytokines can be considered structurally distinct ligands binding different classes of receptors [2]. The cytokine receptors could be classified on the bases of capability to recognize different ligands through their extra- and intra-cellular domains structure: cytokine receptor class 1 superfamily, interferon receptor family, tumor necrosis factor (TNF) receptor family, tumor growth factor (TGF)-β receptor family and IL-8 receptor family [3]. These cytokines exert an important role in immunology, in that they are involved in initiating innate immunity, orchestrating adaptive immune mechanisms and constraining immune and inflammatory responses [2].

The common cytokine receptor γ chain (γc) was first discovered as a component of the IL-2 receptor, which is the prototypical member of the cytokine receptors sharing γc. *IL2RG* gene encoding γc is localized to chromosome Xq13 [4,5]. Mutations in this gene are responsible for the X-linked Severe Combined Immunodeficiency (X-SCID). SCIDs represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T-, B- and Natural Killer- (NK-) cells [6-11] and the X-SCID represents the most common form of SCID, accounting for 40–50% of all cases SCID. In particular, this form of SCID is characterized by the complete absence of both T and NK lymphocytes, whereas B cell number is normal [5].

The receptors containing γc element exert mitogenic effects and regulate lymphocyte development and function [12]. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a proper functionality of γc-dependent cytokines on the whole seems to play prominent roles, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system [13].

Of note, the existence of a previously unappreciated functional interaction between γc and GH-R has been documented. This interaction leads to the activation and intranuclear translocation of STAT5b protein [14]. This relationship was suggested by an initial serendipitous observation on a patient affected with X-SCID, who showed during the follow up a severe impairment of body growth and after the exclusion of a GH deficiency [15,16] was suspected to have an idiopathic short stature and peripheral insensitivity to GH [17,18], similarly to what happens in the presence of GH-R mutation [19]. In this patient, mutational analysis of a few candidate genes, functionally related to short stature and potentially to the immune defect, as GH-R

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itself, JAK2 and STAT5 failed to reveal any further alteration that could explain the GH-R unresponsiveness [17].

This review focuses on the γc as a common transducing element shared between several cytokines and growth hormone receptors, indicating a further functional link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control.

THE RECEPTORS SHARING γC ELEMENT AND INTRACELLULAR TRANSDUCTION SIGNALING

One important family of type I cytokines is the common cytokine receptor γc family, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [3], which plays important roles in immunological functions and in supporting cell survival (Table **1**).

IL-2 is a glycoprotein secreted by activated T lymphocytes, which, in turn, stimulates the development of regulatory T (T_{Reg}) cells and peripheral T cell tolerance [20], as well as the proliferation and apoptosis of activated T cells, with an autocrine effect [21]. Moreover, it promotes the increase of NK cell cytolytic activity and immunoglobulin production by B cells [22].

IL-4 is secreted by T lymphocytes and is required for the development and function of T helper 2 (Th2) cells. In addition, IL-4 also plays a pivotal role in allergy and immunoglobulin class switching. Indeed, a role for IL-4 in B cell Ig class-switch to IgG1 and IgE has been described [23].

IL-7 regulates survival, development and homeostasis of T lymphocytes, both in humans and mice [24,25]. Indeed, alterations of the IL-7-induced signaling apparatus profoundly affects T cell development, as observed in patients with X-SCID [26], as well as in patients with SCID due to mutations in Janus kinase 3 (*JAK3*) [27], or by mutations in *IL7RA* [28]. Moreover, IL-7 is also required for the development of B cells only in mice [29].

Activated $CD4^+$ T cell population is able to produce IL-9, which induces the activation of epithelial cells, B cells, eosinophils and mast cells [30], even though the molecular mechanism activated by this cytockine in T cell remains to be elucidated.

IL-15 induces differentiation of NK cells and is essential for their functionality [31,32]. Defects in IL-15-mediated signaling result in the failure of NK cell development, as reported in X-SCID and JAK3-deficient SCID patients [29]. Furthermore, IL-15 is essential for the homeostatic proliferation of memory $CD8^+$ T cells [25].

Recently a new member of γc sharing cytokine receptors, the IL-21R has been identified [33]. IL-21 has broad actions that include promotion of the terminal differentiation of B cells to plasma cells, cooperation with IL-7 or IL-15 to drive the expansion of $CD8^+$ T cell populations and an action as a pro-apoptotic factor for NK cells, as well as for incompletely activated B cells [25,33]. In addition, it has been reported that the IL-21 promotes the development of type 1 diabetes mellitus [33] and systemic lupus erythematosus (SLE) in animal models [34].

The γc-dependent cytokine receptors use the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway to mediate gene activation or repression [35] (Fig. (**1**)). The JAK/STAT pathway is one of pleiotropic cascades used by mammalian cells to transduce intracellular signals necessary to the development and homeostasis [36]. The binding of the ligand to the specific receptor and the subsequent receptor subunits dimerization leads to the recruitment of JAKs kinases into close proximity of receptors, allowing their trans-phosphorylation and activation. The activated JAKs initiate the intracellular signal transduction through the phosphorylation of tyrosine motifs present in the receptor cytoplasmic domains and in receptor-associated proteins. Four distinct members of JAK family are known in humans: JAK1, JAK2, JAK3 and Tyk2, while seven STAT proteins (STATs) have been identified, named STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [37]. STATs contain a tyrosine residue and also SH2 and SH3 domains that undergo JAK-mediated phosphorylation [38].

All γc-dependent cytokine receptors are able to activate JAK1 and JAK3 proteins, particularly JAK3, which selectively associates with γc [39] and can phosphorylate only STAT3 and STAT5 molecules. Differently, JAK1 can

Table 1. The Biological Roles of Cytokine Receptors Sharing the γc Element

Cytokine	Biological Roles
IL-2	Stimulates the development of T_{Reg} cells, peripheral T cell tolerance, proliferation and apoptosis of activated T cells. Increases NK cell cytolytic activity and immunoglobulin production by B cells
IL-4	Stimulates the development and function of Th2 cells and is implicated in immunoglobulin class switching
IL-7	Regulates survival, development and homeostasis of T lymphocytes both in humans and mice
IL-9	Induces the activation of epithelial cells, B cells, eosinophils and mast cells
IL-15	Regulates differentiation and functionality of NK cells and is essential for the homeostatic proliferation of memory $CD8^+$ T cells
IL-21	Promotes terminal differentiation of B cells to plasma cells and, by cooperating with IL-15, drives the expansion of $CD8^+$ T cell populations. Acts as a pro-apoptotic factor for NK cells

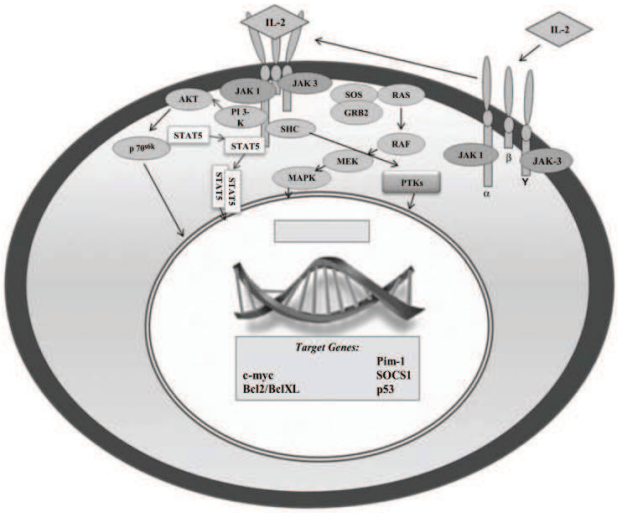

Fig. (1). Intracellular transduction signaling apparatus involving the γc molecule. The γc-dependent cytokine receptors use JAKs kinase signal transducer and STAT proteins to activate the transcription of selected genes that exert mitogenic effects and regulate lymphocyte development, function and survival, such as cyclins, Bcl2/BclXL, p53.

phosphorylate STAT1, STAT3 and STAT5, but only in the presence of an activated JAK3 [40]. Phosphotyrosine-containing motifs in receptor cytoplasmic domains act as docking sites for many signaling proteins, including STATs.

Several molecules are able to activate STAT proteins, including interferons, interleukins and growth factors and hormones. In particular, interferons (IFN)-α/β and IFN-γ lead to a STAT1-mediated anti-viral and anti-bacterial response, growth inhibition, apoptosis and stimulation of tumor suppression [37]. STAT3 is mainly activated by IL-6 and epidermal growth factor (EGF) and is involved in mitogenesis, survival, anti-apoptosis and oncogenesis [41]. STAT4 is predominantly stimulated by IL-12 and is involved in Th1 development in humans. This molecule is also activated by IL-23 in murine cells and, additionally, by IFN-α in human cells, being recruited to type I IFN receptor through interaction with STAT2 [37]. STAT6 molecule is activated by IL-4 and participates in Th2 development. STAT5a and STAT5b are involved in prolactin and growth hormone signaling [42]. STAT3 and STAT5 have been demonstrated to directly participate in tumor development and progression [43,44].

STATs are activated by phosphorylation of conserved tyrosine residue. This event leads to the dissociation of STATs from the receptor, dimerization and acquisition of a high-affinity DNA-binding activity. After the activation, STATs translocate to the nucleus, where they bind to gene promoters and activate transcription of genes involved in cell proliferation, differentiation and survival [36,37,45]. In addition, has been reported the role of STATs molecules in tumor development and progression [46].

Of note, STATs play important roles in oncogenesis by up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators, namely oncogenes, such as Bcl-xL, Mcl-1, cyclins D1/D2, and c-Myc [47]. It has been also documented a constitutive activation of STAT3 or STAT5 in tumor cells resistant to chemotherapeutic agents, that exert their effect on cell apoptosis machinery [43].

Recently, it has also been shown that the inhibition of cytokine-JAK/STAT signaling may be useful for homeostasis and the prevention of chronic inflammation or autoimmunity [48,49]. Proteolysis, dephosphorylation and interaction with inhibitory molecules termed protein inhibitors of activated STATs (PIAS) are responsible for the constitutive inhibitory mechanisms of STATs [50]. Suppressors Of Cytokine Signaling (SOCS) proteins are inhibitory molecules, which down-regulate the receptor expression, by rapid MAPK or PKC-dependent modification [51].

The development of drugs specifically targeting the JAK-STAT regulators or the motifs implicated in such intermolecular interactions might be a potential novel pharmacological therapeutic approach in the therapy of neoplastic disorders.

Recently, we documented that γc is implicated in the control of cell proliferation of malignant hematopoietic cells [52]. Moreover, using of knock-out strategy or through the neutralization of cytokines receptors involving γc in the

signaling apparatus, an anticancer effect has been recently documented [53,54]. This evidence is in keeping with a role of γc as a cofactor in tumor growth. Future research strategies might be designed to evaluate γc levels in different primary leukemic cell lines, in order to compare the amount of the molecule to the type of the tumor or to its aggressiveness. Future studies might clarify whether the levels of protein expression may be used as prognostic factor.

BIOCHEMICAL PATHWAYS INVOLVED IN GROWTH HORMONE RECEPTOR SIGNALING

The GH is a peptide of 191 amino acids and 22 kDa molecular weight, produced by the adenohypophysis, that regulates many important functions such as control of cellular metabolism, immune functions, fertility and somatic growth [55-57]. GH and other mitogenic factors, including hepatocyte growth factor in liver cells, basic fibroblast growth factor in cartilage, epidermal growth factor in kidney, estrogen receptors in the uterus, bone morphogenetic proteins in various tissues, participate to a wide network of well integrated signals [55]. The several functions mediated by GH are activated following GH-R stimulation, the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for Erythropoietin (EPO), Granulocyte colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GMCSF), IL-2 - 7, IL-9, IL-11, IL-12 and many other cytokines [58].

The members of the cytokine receptor superfamily 1 lack intrinsic kinase activity, thus requiring the recruitment of cytoplasmic tyrosine kinases to the intracellular signaling apparatus [59,60]. In particular, JAK2 is required for a fully functional GH-R-mediated pathway [61]. The receptor structure consists of a transmembrane protein with two motifs, an extracellular domain, which binds the ligand and an intracellular domain associated to JAK2 [59].

After the phosphorylation of JAK2, the receptor itself and several intracytoplasmic molecules are promptly phosphorylated on tyrosine residues. Further signaling proteins recruited to JAK2/GH-R complex and/or activated in response to GH include: Shc proteins, that presumably lead to the activation of Ras/mitogen-activated protein kinase (MAPK) pathway [62], insulin receptor substrates, that have been implicated in the activation of phosphatidylinositol-3-kinase (PI3K) and the kinase AKT/protein kinase (PK) B [63], phospholipases, that lead to the formation of diacylglycerol and subsequent activation of PKC and a variety of proteins involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck [64-66].

These observations imply that GH-R, as well as other receptors, is able to integrate different pathways differentially regulated. Several causes of one of these pathways may cause features of altered GHR signaling [67]. It has been shown that different cell types as hepatocytes, fibroblasts and myoblasts have different regulatory mechanisms which lead to cell specific functions of GH [68]. In keeping with this, it has been observed a cell type-restricted STATs activation [69]. STAT5 is not activated following GH stimulation in human fibrosarcoma cells even though these cells express the STAT5 protein [70], thus implying that a selectivity in the involvement of specific STAT subset seems to be a general feature of GH-R signal transduction.

Moreover, it has been documented that GH and IGF-I are able to activate several pathways involved in the control of cell survival and proliferation of lymphoid cells, directly or indirectly through the production of IGF-I [69]. Of note, IGF-I activates a number of biologic effects, as induction of cell growth, maintenance of cell survival by acting on the Bcl family members and induction of cellular differentiation through still poorly characterized mechanisms [71]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factor [70]. Components of the IGF-I system may play a key role in the deregulation of cell cycling or apoptosis in tumor growth [72]. In keeping with this observation, it has been documented that the treatment with GH may increase the risk of developing hematopoietic malignancies, including leukemias or lymphoma [68]. Moreover, evidence suggests a potential role for the GH/IGF-I axis in the development of cancer through the regulation of cell proliferation, differentiation and apoptosis [73]. In addition, it has been documented a positive correlation between circulating IGF-I and IGFBP-3 levels and the risk of developing cancer [74]. Particularly, IGF-I is mitogenic *per se* and exerts an important antiapoptotic effect, whereas IGFBP-3, which is thought to inhibit growth through ligand sequestration, is also supposed to have antiproliferative and proapoptotic effects, thus interfering with tumor growth [74]. Nevertheless, no conclusive data are available of the relationship between the GH/IGF-I axis and the risk of developing cancer.

As matter of fact, progress in defining the pathogenic implications of GH/IGF-I/IGF-I-R and downstream molecules in neoplasia might lead to the development of novel targeting strategies to fight those cancers that may be proven responsive.

THE LINK BETWEEN γC AND GH-R PATHWAYS: THE CONTROL OF CELL GROWTH

The impairment of various GH-induced events in γc deficiencies suggests a potential interaction between GH-R pathways and γc [14]. Particularly, an altered STAT5 phosphorylation following GH-R stimulation in a patient affected with X-SCID and peripheral GH hyporesponsiveness with short stature and delayed bone age, has been described [17]. Allogeneic bone marrow transplantation not only brought along a fully competent immunological reconstitution, but also caused a considerable improvement in linear growth and restored basal and GH-induced levels of insulin growth factor I (IGF-I) [75]. Afterwards, it has been reported an abnormal signal transduction of GH-R in B cell lines (BCLs) from X-SCID patients following GH stimulation. GH-R activation fails to induce phosphorylation on tyrosine residues of STAT5 and its nuclear translocation, thus no functional effect is observed in these cells [14]. Of note, *IL2RG* gene transduction of X-SCID cells with WT γc gene, restored GH induced proliferation and STAT5 nuclear translocation [14].

Finally, a direct role of γc in the regulation of cell cycle progression and in the control of both GH-induced cell and spontaneous growth has been proposed on the basis of γc silencing experiments. Effects of GH stimulation in γc-

silenced or X-SCID BCLs have been compared with control BCLs. In γc-silenced or X-SCID BCLs, recombinant GH induced proliferation at a much lower extent, while no change in subcellular redistribution of STAT5 has been observed. In control BCLs, GH stimulation determines a rapid increase of nuclear amount of STAT5 and enhances proliferation. Thus, the γc-silencing results in the severe decline of self-sufficient growth in BCLs, as shown by comparing the ability to proliferate of γc-silenced BCLs and control cells [76]. In addition, the activation of JAK3 is a downstream event of γc activation and a correlation between γc amount and the extent of constitutive activation of JAK3 has been associated to autonomous cell growth and malignant transformation of lymphoid cells [76]. Of note, a relationship between γc expression and the amount of constitutively activated JAK3 has been shown. In particular, a higher constitutive activation of JAK3 was found in control BCLs, whereas a decrease in phospho-JAK3 levels was observed in γc-silenced and in X-SCID BCLs, despite a comparable amount of the whole protein. Moreover, the amount of constitutive JAK3 paralleled the amount of γc [76]. These data suggest that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression.

Recently, clinical gene-therapy trials using ex-vivo retroviral vectors, have been proven as a corrective therapeutic approach for X-SCID in humans [77]. In particular, immunological reconstitution has been documented in 17 out of 20 patients enrolled in two distinct clinical trials through gene therapy approach [77]. Unfortunately, 5 of these patients developed a lymphoproliferative disorder [78]. Even though it has been reported that retroviral integration of the corrective IL2RG occurred near the locus of LMO2 oncogene and may have upregulated LMO2 expression [79], further studies documented that leukemogenesis was not due to insertional mutagenesis and raised the possibility that IL2RG may be oncogenic *per se* [80]. In addition, proliferation and transformation of normal hematopoietic and laeukemic cells has been observed with supra-physiological doses of GH [81]. The risk of developing cancer is determined by a combination of genetic factors and environmental effects, in particular diet and lifestyle. There is increasing evidence that the GH/IGF-I axis may provide a link between these factors. GH and related signaling molecules, through their implication in the regulation of normal cell proliferation, differentiation and apoptosis, may act as a cofactor in the overgrowth of cancer cells [73]. Therefore, the evidence that these molecules may be implicated in the control of tumor progression highlights the need to go further in a detailed understanding of the mechanisms underlying the process aimed to identifying new potential therapeutic targets.

CONCLUSIONS

Immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion [68]. Several evidences demonstrate the existence of a previously unappreciated relationship between distinct elements, such as GH-R and γc and their signaling pathways. Crosstalk between receptor signaling systems is now emerging as an important and exciting area of signaling research.

The impairment of various GH-induced events in γc deficiencies suggests a potential interaction between GH-R pathways and γc, indicating a further functional link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. The existence of shared molecular mechanisms of transduction between immune and endocrine systems is well documented in several autoimmune disorders characterized by both endocrine and immune features [82-85]. GH-R pathways and γc interaction leads to the activation and intranuclear translocation of STAT5b protein. The signals mediated by STATs generally play a central role in the control of important cellular events such as cell proliferation, differentiation and apoptosis [46], even though the overall role of the STAT molecules in GH-R signal transduction has not been fully elucidated. The overall signal transduction properties of GH-R in X-SCID patients and control BCLs following GH stimulation, have been recently elucidated. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in γc negative patients cell lines in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with WT γc gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γc in GH-R signaling. Of note, a co-localization of GH-R and γc has been documented. Even though a physical interaction between the two proteins may be possible, any physical association has not yet been documented [14]. Currently, studies are ongoing to identify potential physical interactions between the two proteins. Moreover, evidence suggests that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression. Whether the participation of γc to the GH-R confers some additional properties to the receptor in hematopoietic cell differentiation and functioning remains to be elucidated.

DISCLOSURE

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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REFERENCES

[1] Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994; 28: 253-62.

[2] Ô'Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity 2012; 36: 542-50.

[3] Taga T, Kishimoto T. Cytokine receptors and signal transduction. FASEB J 1993; 7: 3387-96.

[4] Leonard WJ. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. Annu Rev Med 1996; 47: 229-39.

[5] Notarangelo LD, Giliani S, Mazza C, *et al.* Of genes and phenotypes: the immunological and molecular spectrum of combined immune deficiency. Defects of the gamma(c)-JAK3 signaling pathway as a model. Immunol Rev 2000; 178: 39-48.
[6] Fischer A, Cavazzana-Calvo M, De Saint Basile G, *et al.* Naturally occurring primary deficiencies of the immune system. Annu Rev Immunol 1997; 15: 93-124.
[7] Aloj G, Giardino G, Valentino L, *et al.* Severe combined immunodeficiences: new and old scenarios. Int Rev Immunol 2012; 31: 43-65.
[8] Granados EL, Porpiglia AS, Hogan MB, *et al.* Clinical and molecular analysis of patients with defects in μ heavy chain gene. J Clin Invest 2002; 110(7): 1029-35
[9] De Vries E, Alvarez Cardona A, Abdul Latiff AH, *et al.* Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol 2012; 167: 108-19.
[10] Markert ML, Marques J, Neven B, *et al.* First use of thymus transplantation therapy for Foxn1 deficiency (nude/SCID): a report of two cases. Blood 2011; 117: 688-96.
[11] Palamaro L, Vigliano I, Giardino G, *et al.* SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. J Investig Allergol Clin Immunol 2012; 22: 67-70.
[12] Sugamura K, Asao H, Kondo M, *et al.* The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. Ann Rev Immunol 1996; 14: 179-205.
[13] Lindemann MJ, Benczik MGaffen SL. Anti-apoptotic signalling by the interleukin-2 receptor reveals a function for cytoplasmic tyrosine residues within the common gamma (gamma c) receptor subunit. J Biol Chem 2003; 278: 10239-49.
[14] Adriani M, Garbi C, Amodio G, *et al.* Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus. J Immunol 2006; 177: 6889-95.
[15] Cornelli G, Di Somma C, Prodam F, *et al.* Cut-off limits of the GH response to GHRH plus arginine test and IGF-I levels for the diagnosis of GH deficiency in late adolescents and young adults. Eur J Endocrinol 2007; 157(6): 701-8.
[16] De Bellis A, Salerno M, Conte M, *et al.* Antipituitary antibodies recognizing growth hormone (GH)-producing cells in children with idiopathic GH deficiency and in children with idiopathic short stature. J Clin Endocr Metab 2006; 91: 2484-9.
[17] Ursini MV, Gaetaniello L, Ambrosio R, *et al.* Atypical X-linked SCID phenotype associated with growth hormone hyporesponsiveness. Clin Exp Immunol 2002; 129: 502-9.
[18] Salerno MC, Balestrieri B, Matrecano E, *et al.* Abnormal GH receptor signaling in children with idiopathic short stature. J Clin Endocr Metab 2001; 86: 3882-8.
[19] Goddard AD, Covello R, Luoh SM, *et al.* Mutations of the growth hormone receptor in children with idiopathic short stature. N Engl J Med 1995; 333: 1093-8.
[20] Sakaguchi S, Yamaguchi T, Nomura T, *et al.* Regulatory T cells and immune tolerance. Cell 2008; 133: 775-87.
[21] D'Souza WN, Lefrançois L. IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. J Immunol 2003; 171: 5727-35.
[22] Kim HP, Imbert J, Leonard WJ. Both integrated and differential regulation of components of the IL-2/IL-2 receptor system. Cytokine Growth Factor Rev 2006; 17: 349-66.
[23] Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nature 2008; 8: 218-30.
[24] Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. Nature 2007; 7: 144-54.
[25] Surh CD, Sprent J. Homeostasis of Naive and Memory T Cells. Immunity 2008; 29: 848-62.
[26] Noguchi M, Yi H, Rosenblatt HM, *et al.* Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 1993; 73: 147-57.
[27] Macchi P, Villa A, Giliani S, *et al.* Mutation of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature 1995; 377: 65-8.
[28] Puel A, Ziegler SF, Buckley RH, *et al.* Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet 1998; 20: 394-7.
[29] Leonard WJ. Cytokines and immunodeficiency diseases. Nat Rev Immunl 2001; 1: 200-8.
[30] Nowak EC, Noelle RJ. Interleukin-9 as a T helper type 17 cytokine. Immunology 2010; 131: 169-73.
[31] Lodolce JP, Boone DL, Chai S, *et al.* IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. Immunity 1998; 9: 669-76.
[32] Kennedy MK, Glaccum M, Brown SN, *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med 2000; 191: 771-80.
[33] Spolski RLeonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol 2008; 26: 57-79.
[34] Bubier JA, Sproule TJ, Foreman O, *et al.* A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. Proc Natl Acad Sci USA 2009; 106: 1518-23.
[35] Demoulin JB, Renauld JC. Signalling by cytokines interacting with the interleukin-2 receptor gamma chain. Cytokines Cell Mol Ther 1998; 4: 243-56.
[36] Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci 2004; 117: 1281-3.
[37] Ivashkiv LB, Hu X. Signaling by STATs. Arthritis Res Ther 2004; 6: 159-68.
[38] Darnell JEJ. STATs and gene regulation. Science 1997; 227: 1630-5.
[39] Miyazaki T, Kawahara A, Fujii H, *et al.* Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. Science 1994; 266: 1045-7.
[40] Witthuhn BA, Williams MD, Kerawalla H, *et al.* Differential substrate recognition capabilities of Janus family protein tyrosine kinases within the interleukin 2 receptor (IL2R) system: Jak3 as a potential molecular target for treatment of leukemias with a hyperactive Jak-Stat signaling machinery. Leuk Lymphoma 1999; 32: 289-97.
[41] Inghirami G, Chiarle R, Simmons WJ, *et al.* New and Old Functions of STAT3. Cell Cycle 2005; 49: 1131-3.
[42] Ambrosio R, Fimiani G, Monfregola J, *et al.* The structure of human STAT5A and B genes reveals two regions of nearly identical sequence and an alternative tissue specific STAT5B promoter. Gene 2002; 285: 311-8.
[43] Bowman T, Garcia R, Turkson J, *et al.* STATs in oncogenesis. Oncogene 2000; 19: 2474-88.
[44] Moriggl R, Topham DJ, Teglund S, *et al.* Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 1999; 10: 249-59.
[45] O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002; 109 Suppl: S121-31.
[46] Calu V, Migliavacca M, Bazan V, *et al.* STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol 2003; 197: 157-68.
[47] Lockyer HM, Tran E, Nelson BH. STAT5 is essential for Akt/p70S6 kinase activity during IL-2-induced lymphocyte proliferation. J Immunol 2007; 179: 5301-8.
[48] Wu T, Mohan C. The AKT axis as a therapeutic target in autoimmune diseases. Endocr Metab Immune Disord Drug Targets 2009; 9: 145-50.
[49] Xiong Z, Ma A, Chen H. JAK3 inhibitors in organ transplantation and autoimmune disease. Recent Pat Inflamm Allergy Drug Discov 2010; 4: 75-81.
[50] Greenhalgh CJ, Hilton DJ. Negative regulation of cytokine signaling. J Leukoc Biol 2001; 70: 348-56.
[51] Flores-Morales A, Greenhalgh CJ, Norstedt G, *et al.* Negative regulation of growth hormone receptor signaling. Mol Endocrinol 2006; 20: 241-53.
[52] Vigliano I, Palamaro L, Bianchino G, *et al.* Role of the common g chain in cell cycle progression of human malignant cell lines. Int Immunol 2012; 24: 159-67.
[53] Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol 2009; 9: 480-90.
[54] Rosenberg SA. Interleukin-2 and the development of immuno-therapy for the treatment of patients with cancer. Cancer J Sci Am 2000; 6: S2-S7.
[55] Waters MJ, Hoang HN, Fairlie DP, *et al.* New insights into growth hormone action. J Mol Endocrinol 2006; 36: 1-7.

[56] Salerno M, Esposito V, Farina V, *et al.* Improvement of cardiac performance and cardiovascular risk factors in children with GH deficiency after two years of GH replacement therapy: an observational, open, prospective, case-control study. J Clin Endocr Metab 2006; 91: 1288-95.

[57] Salerno M, Esposito V, Capalbo D, *et al.* Metabolic effects of growth hormone (GH) deficiency and GH replacement therapy in children and adolescents. J Pediatr Endocrinol Metab 2007; 20: 345-50.

[58] Zhu T, Goh ELK, Graichen R, *et al.* Signal transduction via the growth hormone receptor. Cell Signal 2001; 13: 599-616.

[59] Fuh G, Mulkerrin MG, Bass S, *et al.* The human growth hormone receptor. J Biol Chem 1990; 265: 3111-5.

[60] Han Y, Leaman DW, Watling D, *et al.* Participation of JAK and STAT proteins in growth hormone-induced signaling. J Biol Chem 1996; 271: 5947-52.

[61] Argetsinger LS, Campbell GS, Yang X, *et al.* Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 1993; 74: 237-44.

[62] Vanderkuur JA, Butch ER, Warters SB, *et al.* Signaling molecules involved in coupling growth hormone receptor to mitogen-activated protein kinase activation. Endocrinolgy 1997; 138: 4301-7.

[63] Yokota I, Hayashi H, Matsuda J, *et al.* Effect of growth hormone on the translocation of GLUT4 and its relation to insulin-like and anti-isulin action. Biochim Biophys Acta 1998; 1404: 451-6.

[64] Zhu T, Goh ELK, Lobie PE. Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. J Biol Chem 1998; 273: 10682-9.

[65] Zhu T, Goh ELK, LeRoith D, *et al.* Growth hormone stimulates the formation of a multiprotein signaling complex involving $p130^{Cas}$ and CrkII. J Biol Chem 1998; 273: 33864-75.

[66] Pignata C, Prasad KVS, Hallek M, *et al.* Phosphorylation of src family lck tyrosine kinase following interleukin-12 activation of human natural killer cells. Cell Immunol 1995; 165: 211-6.

[67] Capalbo D, Melis D, De Martino L, *et al.* Noonan-like syndrome with loose anagen hair associated with growth hormone insensitivity and atypical neurological manifestations. Am J Med Genet 2012; 158A: 856-60.

[68] Jeay S, Sonenshein GE, Postel-Vinay MC, *et al.* Growth hormone can act as a cytokine controlling survival and proliferation of immune cells: new insights into signaling pathways. Mol Cell Endocrinol 2002; 188: 1-7.

[69] Baixeras E, Jeay S, Kelly PA, *et al.* The proliferative and antiapoptotic actions of growth hormone and insulin-like growth factor-1are mediated through distinct signaling pathways in the Pro-B Ba/F3 cell line. Endocrinology 2000; 142: 2968-77.

[70] Firth SM, Baxter RC. Cellular actions of the Insulin-like growth factor binding proteins. Endocr Rev 2002; 23: 824-54.

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[71] Cohen AC, Nadeau KC, Tu W, *et al.* Cutting edge: decreased accumulation and regulatory function of CD4+CD25(high) T cells in human STAT5b deficiency. J Immunol 2006; 177: 2770-4.

[72] Yakar S, Pennisi P, Kim CH, *et al.* Studies involving the GH-IGF axis: lessons from IGF-I and IGF-I receptor gene targeting mouse models. J Endocrinol Invest 2005; 28: 19-22.

[73] Jenkins PJ, Bustin SA. Evidence for a link between IGF-I and cancer. Eur J Endocrinol 2004; 151: S17-S22.

[74] Renehan AG, Zwahlen M, Minder C, *et al.* Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004; 363: 1346-53.

[75] Salerno MC, Busiello R, Esposito V, *et al.* Allogenic bone marrow transplantation restores IGF-I production and linear growth in a gamma-SCID patient with abnormal growth hormone receptor signaling. Bone Marrow Transplant 2004; 33: 773-5.

[76] Amorosi S, Russo I, Amodio G, *et al.* The cellular amount of the common gamma-chain influences spontaneous or induced cell proliferation. J Immunol 2009; 182: 3304-9.

[77] Cavazzana-Calvo M, Carlier F, Le Deist F, *et al.* Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. Blood 2007; 109: 4575-81.

[78] Alton E, Ferrari S, Griesenbach U. Progress and prospects: gene therapy clinical trials (Part 1). Gene Ther 2007; 14: 1439-47.

[79] Davé UP, Akagi K, Tripathi R, *et al.* Murine leukemias with retroviral insertions at Lmo2 are predictive of the leukemias induced in SCID-X1 patients following retroviral gene therapy. PloS Genet 2009; 5: e1000491.

[80] Woods NB, Bottero V, Schmidt M, *et al.* Therapeutic gene causing lymphoma. Nature 2006; 440: 1123.

[81] Ogilvy-Stuart AL, Gleeson H. Cancer risk following growth hormone use in childhood: implications for current practice. Drug Saf 2004; 27: 369-82.

[82] Mazza C, Buzi F, Ortolani F, *et al.* Clinical heterogeneity and diagnostic delay of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome. Clin Immunol 2011; 139: 6-11.

[83] Capalbo D, Fusco A, Aloj G, *et al.* High intrafamilial variability in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy: a case study. J Endocrinol Invest 2012; 35: 77-81.

[84] Capalbo D, Mazza C, Giordano R, *et al.* Molecular background and genotype-phenotype correlation in autoimmune-polyendocrinopathy-candidiasis-ectodermal-distrophy patients from Campania and in their relatives. J Endocrinol Invest 2012; 35: 169-73.

[85] Busiello R, Adriani M, Locatelli F, *et al.* Atypical features of familial hemophagocytic lymphohistiocytosis. Blood 2004; 103: 4610-12.

2.5. Conclusive remarks

Immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion (139). Evidence documented that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression (184), suggesting the interplay between endocrine and immune system and the main role of γc to regulate cell proliferation and growth.

Alterations of the *IL2RG* gene leads to X-linked Severe Combined Immunodeficiency (X-SCID), which is characterized by an absence of T cells and natural killer cells. Patients with X-SCID have a normal number of B cells but these are non-functional. Gene therapy trials, as new therapeutic approach for X-SCID, have been performed leading to immunological reconstitution but, unfortunately, the occurrence of leukemia was documented in few patients (139). In 2 cases it was associated with an insertional mutagenesis in *LMO2* oncogene. In mice, it has been reported that *IL2RG* cooperates with *LMO2* in inducing hematopoietic tumors (210). Of note, no clonal lymphoproliferation has been reported, in patients receiving gene therapy for ADA deficiency, despite the integration sites near *LMO2* (140). Moreover, overexpression of γc, in X-SCID mice, led to T-cell lymphomas and thymic hyperplasia with no common integration site documented. These results suggested that insertional mutagenesis was not the only cause of leukemogenesis, raising the possibility that *IL2RG* is oncogenic *per se*.

In this context, we demonstrated that γc exerts a role in either spontaneous or GH-induced cell cycle progression, and this regulatory effect depends on the amount of protein expression (174). Moreover, we documented an over-expression of γc protein in

continuous malignant hematopoietic cell lines, and a directly correlation between the amount of molecule with spontaneous cell growth was found. In addition, an increased expression of all D-type cyclins and a direct correlation between the amount of γc and cyclins A2 and B1 expression was documented, thus implying a critical regulatory role of γc on cell proliferation of continuous malignant hematopoietic cells (175).

Recently, we found a predominant and selective up-regulation of *IL2RG* expression levels in B-pre ALL cells, differently from what expect because it is well documented the role of γc-signaling cascade in physiological T-cell development and evidence of involvement of γc in biology of T-ALL are available (176). Moreover, exploring the potential mechanism by which γc regulates leukemia proliferation and survival in B-pre ALL cells, we observed that the γc promoted cell cycle progression through the upregulation of D-type cyclins, while it allowed cell survival *via* autophagy, as suggested by the up-regulation of BECLIN-1 in B-pre ALL cells.

The new knowledge on the molecular basis of leukemogenesis will allow the definition of new scenarios either to identify novel prognostic biomarkers or to develop new therapeutic strategies for human hematological malignancies. Currently, several studies exploring the role of several genes and proteins involved in leukemogenesis are available. The results came out from our studies will give a great contribution in this field by unraveling still unidentified mechanisms involved in the pathogenesis of leukemia and potential novel clinical implications. In particular, the evidence that γc plays a role in the growth, cell cycle progression and cell proliferation in primary malignant cell lines will be useful at identifying specific drugs targeted to the molecule that could exert some beneficial effect on the tumor growth and progression of the disease through the modulation of the γc expression and activation. Moreover, the

selective up-regulation of γc expression in specific subtypes of leukemia could be a valuable tool for innovative therapeutic approach for B-pre ALL.

CHAPTER III

"Predisposition to cancer and autoimmunity associated with PIDs"

Immunedeficiencies are genetic or acquired diseases showing predisposition to severe infections, but also, in certain circumstances, autoimmunity and cancer, caused to abnormal functionality of immune system.

The increased risk of developing malignancy of patients with immunodeficiencies is caused to a defective immunity towards cancer cells. The overall risk for children with congenital immunodeficiency to develop tumor is approximately 4-25% (211). The type of malignancy is highly dependent on the primary immunodeficiency, the age of the patient and probably viral infection, suggesting that different pathogenetic mechanisms are implicated in each case. Of note, non-Hodgkin's lymphomas predominate (60% of cases) (211). Nevertheless, only a few studies regarding the incidence of malignancy in primary immunedeficiencies, the histopathological types and prognosis are so far available. It has been documented that the median age of diagnosis is 7.1 years with a male predominance, due to an increased frequency of X-linked disorders. Moreover, it has been shown that the diseases more often associated with tumor were CVID, WAS, A-T and SCID (212).

The immune system has the peculiar ability to monitor and control infections from non-self pathogens while maintaining tolerance to self-antigens. The establishment and maintenance of the mechanism self-tolerance is a necessary ability of adaptive immunity. Breakdown of either central or peripheral tolerance can lead to autoimmunity. In PIDs the occurrence of autoimmunity represents a failure of immune system in responding to non-self pathogens and simultaneously a vigorous reaction to

self-antigens. Of note, studies of several monogenic human immune disorders and animal models have shown that the impaired functionality of the immune system can provoke breakdown of central and peripheral tolerance and consequently the development of autoimmune disease.

3.1. Ataxia telangiectasia

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder characterized by progressive neurological dysfunction, especially affecting the cerebellum, oculocutaneous telangiectasia, immunodeficiency, high incidence of neoplasms and hypersensitivity to ionizing radiations (213, 214). A-T is associated with mutations in the ATM gene, spanning 150 kb of genomic DNA and encoding for a 370-kDa serine/threonine (215). ATM plays a key role in the control of various cellular processes such as DNA repair, cell cycle progression, gene transcription, protein synthesis and degradation, and apoptosis. A-T may be considered a prototype of the DNA repair defect syndromes, since the disease shows the typical consequences of defects in the DNA damage response (DDR): degeneration of specific tissue affecting particularly the nervous and immune systems, chromosomal instability, and sensitivity to specific DNA-damaging agents. However, the neuronal degeneration in A-T is only partially due to defective DNA damage response DDR (216). In fact, it has been proposed a new role of ATM in maintaining the reducing power of the cellular environment, acting as redox sendor. Furthermore, recent studies highlight the role of ATM in modulating mitochondrial homeostasis. Of note, a fraction of ATM localizes to the mitochondria and can be activated by mitochondrial dysfunction in the absence of DNA damage

(217). Since the high rate of transcription and translation in neurons creates a stressful environment, neuronal genomic and mitochondrial DNA are constantly attacked, suggesting that the mitochondrial dysfunction and the increase in mitochondrial reactive oxygen species may contribute to the A-T phenotype.

To date, only supportive care aimed to halt the progressive neurodegeneration is available for the treatment. Recently, an improvement of neurological signs during short-term treatment with oral betamethasone has been reported. In A-T, and in several neurodegenerative disorders, an inappropriate apoptosis seems to play an important pathogenic role. In this contest, studies showed that Glucocorticoids (GCs) were able to enhance apoptosis in inflammatory and immune cells, whereas, at the same, time they seem to protect tissues in which the inflammation takes place (218, 219). GCs were capable to diffusing into the Central Nervous System (CNS) by crossing the blood-brain barrier (BBB), where they exerted effects on the suppression of inflammation or as antioxidant and protected post-mitotic neurons from apoptosis. Eventually, GCs potentially also played a role in modulating synaptic plasticity, reducing the impact of disability (220).

However, the molecular and biochemical mechanisms by which the steroid produces such effects have not yet been elucidated. A better understanding of the mechanisms of action of GCs in the brain is needed, because in A-T during the initial phase of cell loss, the neurological impairment may be rescued by interfering the biochemical pathways. This would open a new window of intervention in this so far non-curable disease.

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REVIEW ARTICLE

Betamethasone therapy in Ataxia Telangiectasia: unraveling the rationale of this serendipitous observation on the basis of the pathogenesis

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Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder characterized by progressive neurological dysfunction. To date, only supportive care aimed to halt the progressive neurodegeneration is available for the treatment. Recently, an improvement of neurological signs during short-term treatment with betamethasone has been reported. To date, the molecular and biochemical mechanisms by which the steroid produces such effects have not yet been elucidated. Therefore, a review of the literature was carried out to define the potential molecular and functional targets of the steroid effects in A-T. Glucocorticoids (GCs) are capable to diffusing into the CNS by crossing the blood–brain barrier (BBB) where they exert effects on the suppression of inflammation or as antioxidant. GCs have been shown to protect post-mitotic neurons from apoptosis. Eventually, GCs may also modulate synaptic plasticity. A better understanding of the mechanisms of action of GCs in the brain is needed, because in A-T during the initial phase of cell loss, the neurological impairment may be rescued by interfering the biochemical pathways. This would open a new window of intervention in this so far non-curable disease.

Introduction

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder that affects several body systems and tissues leading to a complex and severe phenotype [1,2]. The hallmark of A-T is the progressive neurological dysfunction characterized by uncoordinated and ataxic movements as a result of cerebellar atrophy or dysfunction [3]. Other features of A-T include telangiectasia, immune and endocrine dysfunctions, cellular radiosensitivity, genomic instability, premature aging, and predisposition to cancer [3,4]. The prognosis for survival is poor, and death usually occurs during about the second or third decade of life predominantly caused by the progressive neurodegeneration, pulmonary failure with or without identifiable pneumonia, or cancer [5]. Currently, there is no effective treatment for A-T, but only supportive care aimed to halt progressive neurodegenerative changes. In a recent study, we reported on the amelioration of neurological signs, assessed by Scale for the Assessment and Rating of Ataxia (SARA), during short-term treatment with oral betamethasone [6,7]. Far from being a definitive therapeutic approach, this observation makes us able to argue that, during the initial phase of cell loss, biochemical and functional cerebellar abnormalities may still be modified, by interfering the biochemical pathways.

As for the intimate mechanism implicated in such beneficial effect of the betamethasone, no conclusive information is available. The central issue is to clarify whether this improvement is related to the peripheral effect of the drug or rather to an effect of the drug on the central nervous system (CNS) performance activity. On peripheral blood mononuclear cells, an effect of betamethasone on radical oxygen species (ROS) production has been documented [8], and evidence indicates that steroids may cross the blood–brain barrier (BBB). In this review, we will focus on new aspects of pathophysiology and their implication in understanding the mechanism of action of betamethasone in A-T.

ATM defect and A-T pathogenesis

A-T is associated with mutations in the ATM gene encoding for a 370-kDa serine/threonine kinase, which

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shares sequence similarities with the catalytic subunit of phosphatidyl inositol-3 kinase. The ATM gene is large, spanning 150 kb of genomic DNA and encoding a ubiquitously expressed transcript of approximately 13 kb [9]. The classic form of A-T results from the presence of two truncating ATM mutations, leading to total loss of function of the ATM protein, whereas milder forms are associated with a leaky splice site ATM mutation or with mis-sense mutations. The presence of these latter alterations may allow some expression of mutant ATM with a degree of residual kinase activity [10].

ATM plays a key role in the control of various cellular processes such as DNA repair, cell cycle progression, gene transcription, protein synthesis and degradation, and apoptosis (Fig. 1). The disease may be considered a prototype of the DNA repair defect syndromes. In fact, ATM represents the central component of the signal transduction pathway responding to double-strand DNA breaks (DSBs) caused by IR, endogenous and exogenous DNA-damaging agents (Fig. 1) [11]. A-T demonstrates the typical consequences of defects in the DNA damage reponse (DDR): degeneration of specific tissue affecting particularly the nervous and immune systems, chromosomal instability, and sensitivity to specific DNA-damaging agents. However, the neuronal degeneration in A-T is only partially due to defective DNA damage response DDR [12] and recognizes a complex pathogenesis. It has been recently proposed that ATM also acts as a redox sensor. ATM activation may derive directly from oxidative stress or DNA damage induced by oxidative stress. In the first case, a disulfide bridge is formed between cysteine (C2991) residues in an ATM dimer [13], whilst in the second case an active monomer forms from an inactive dimer [14]. This evidence suggests a role of ATM in signaling other than direct DNA damage. Furthermore, recent studies highlight the role of ATM in modulating mitochondrial homeostasis. A fraction of ATM localizes to the mitochondria and can be activated by mitochondrial dysfunction in the absence of DNA damage [15]. The high rate of transcription and translation in neurons creates a stressful environment, where neuronal genomic and mitochondrial DNA are constantly attacked. Mitochondrial dysfunction has been found to be implicated in a significant number of neurological diseases, including Parkinson's disease, Huntington's

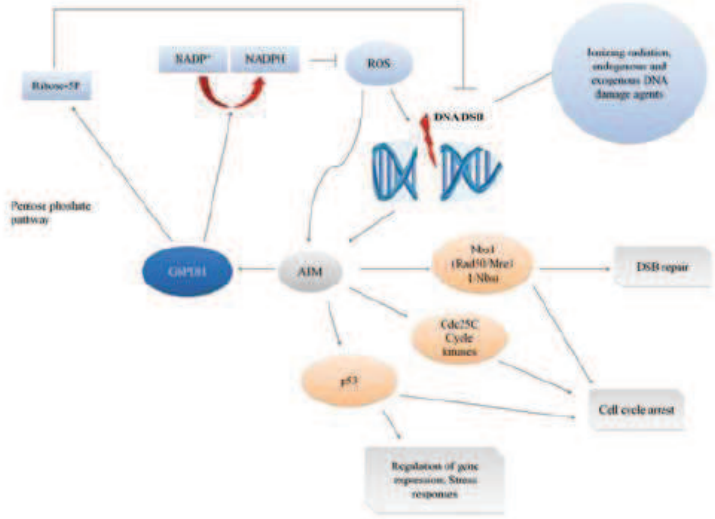

Figure 1 Following the induction of double-strand DNA breaks (DSB), A-T mutated (ATM) is activated, and a portion of nuclear ATM binds to the DSB sites. Activated ATM is then able to phosphorylate substrates that are involved in a series of events including cell cycle checkpoint activation and apoptosis. ATM-dependent phosphorylation of p53 and Chk2 can inhibit the cell cycle at various phases. ATM also phosphorylates NBS1, which is involved in the intra-S-phase checkpoint. ATM plays a key role also in maintaining the reducing power of the cellular environment upregulating pentose phosphate pathway (PPP), which converts glucose-6-phosphate to ribose-5-phosphate. In this process, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) reduce NADP to NADPH. High levels of ROS, under normal conditions, activate ATM, which, in turn, promotes G6PD activity, thus restoring the redox state of the cells. Oxidative stress contributes to the accumulation of DNA damage, leading to ATM activation. However, ATM oxidation directly induces its activation in the absence of DSBs.

disease, Alzheimer's disease, amyotrophic lateral sclerosis, and various peripheral neuropathies, as well as the normal aging process [16,17]. These findings suggest that the mitochondrial dysfunction and the increase in mitochondrial reactive oxygen species may contribute to the A-T phenotype.

DNA repair defect, and neurodegeneration

A-T provides a well-characterized example of the relationships that exist between repair defects and neurodegenerative disease.

The high rates of transcription and translation in neurons are associated with high rates of metabolism and mitochondrial activity. The amount of oxygen consumed by the brain relative to its size far exceeds that of other organs. This high activity creates a stressful environment where neuronal genomic and mitochondrial DNA are constantly attacked by damaging metabolic by-products, primarily ROS [18]. Because neurons are post-mitotic cells and, in case they are irreversibly damaged, they cannot be replaced, they should survive as long as the organism does. Thus, they have elaborated, stringent defense mechanisms to ensure their longevity [19], such as DDR, an elaborate signaling network activated by DNA damage [20,21]. Genetic deficiencies in enzymes that detect or repair DNA damage can induce the apoptosis of specific neuronal populations or further sensitize them to genotoxic stresses [22]. Accumulation of DNA damage in neurons is one of the major forms of damage involved in brain aging and neurodegeneration. Many neurodegenerative syndromes result from defective DNA strand break responses. However, the disease-specific hallmarks of A-T with regard to the onset and course of neurodegeneration likely reflect selective DDR requirements in the specific areas of CNS [23]. ATM signaling functions predominantly in recently post-mitotic neurons [24] to trigger apoptosis of cells, which have experienced excess DNA damage during brain development. A failure to do this may result in the establishment of genetic lesions that eventually result in cell loss and neurodegeneration [25]. According to this model, in the absence of ATM, damaged neurons survive and populate the Purkinje neuron layer, only to degenerate later as a result of DNA damage experienced during their development.

Autoptical and bioptical studies show atrophy of the cerebellar folia, widespread loss of Purkinje cells, granule cell loss, and significant thinning of the molecular layer of the cerebellum. Purkinje cells have abnormally smooth dendrites with reduced arborizations, and they are often displaced in the molecular layer of the cerebellum [26]. Because basket cell interneurons are present in the cerebellum, it is likely that the Purkinje cell layer, initially formed, is later followed by progressive degeneration. Even though the neurodegenerative phenotype in younger A-T patients is restricted to Purkinje and granule cells of the cerebellum, it broadens considerably with age and may extend to the basal ganglia. This widespread degeneration results in a progressive decline in neural function [23].

ATM and oxidative stress

ATM appears to be involved in the response to oxidative stress [27], possibly acting as a sensor of ROS [13,28]. The generation of ROS can arise either from toxic insults or from normal metabolic processes. The overproduction of oxidants 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 [29]. In mammalian cells, several defense mechanisms against oxidative damage have been documented [30,31]. The first one is based on low molecular weight antioxidants or protein scavengers [32] such as glutathione, which appears in both its oxidized (GSSG) and reduced (GSH) forms [32]. The second one includes antioxidant enzymes like superoxide dismutases, SOD1 and SOD2, glutathione peroxidase, catalase [29], and cytochrome p450 reductase [33].

The CNS is particularly vulnerable to oxidative stress due to the high rate of metabolism, the disproportionately low levels of oxidative defense mechanisms, and the high content of easily oxidized substrates. In this context, an increased and unopposed ROS production can lead to neurotoxicity resulting in neural damage and eventually cell death [29]. Evidences support the direct relationship between excessive ROS production and the pathogenesis of A-T [34]. $ATM^{-/-}$ cells exhibit high concentrations of ROS and hypersensitivity to agents that induce oxidative stress [32], and A-T lymphoblasts reduce glutathione more slowly than normal cells after glutathione depletion induced by oxidative stress [35]. However, other works failed to identify an impaired GSH biosynthesis in cultured A-T cells [36,37]. Moreover, recent studies have documented the presence of high levels of oxidative damage in patients with A-T [8,38], confirming previous observations in mouse models [39]. We also observed in a group of patients with A-T the existence of an inverse correlation between the degree of cerebellar atrophy and GSH levels [8]. Furthermore, in ATM-deficient mice, the overexpression of SOD1 exacerbated certain features of the A-T

phenotype [40]. These observations suggest that the impaired response to ROS in A-T cells might influence neuronal survival [41].

ATM deficiency causes oxidative damage to proteins and lipids in brain, testes, and thymus, genomic instability and hypersensitivity to IR and other treatments that generate ROS [34]. A recent study shows that ATM oxidation directly induces its activation in the absence of DSBs [42], confirming that ATM acts as sensor of ROS in human cells [42].

ATM plays a key role in maintaining the reducing power of the cellular environment upregulating pentose phosphate pathway (PPP), which converts glucose-6-phosphate to ribose-5-phosphate [41]. In this process, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) reduce NADP to NADPH [43]. High levels of ROS, under normal conditions, activate ATM, which, in turn, promotes G6PD activity, thus restoring the redox state of the cells. In patients with A-T, this feedback might be compromised, leading to the accumulation of ROS [41]. Similar to ATM-deficient cells, G6PD-deficient cells are more sensitive to apoptosis induced by IR [44].

It has been observed that ATM activation increases G6PD favoring the interaction between heat shock protein (HSP) 27 and G6PD [41]. ATM in fact induces serine 78 phosphorylation of HSP27 and mediates G6PD activation. It is possible that this phosphorylation increases HSP27 affinity for G6PD increasing, in turn, its activity.

A-T and steroid therapy

Currently, there is no effective treatment for A-T. A single case report pointed out that steroids produced in a child a short-term improvement in ataxia [45]. Recent clinical reports extended this clinical observation and documented a clear-cut beneficial effect of such therapy that was inversely correlated with the extent of cerebellar atrophy. This beneficial effect was also inversely correlated with the age of the patients [8]. Of note, this effect was strictly drug dependent, in that the drug withdrawal paralleled the worsening of the neurological signs [6]. A recent double-blind, randomized, placebo-controlled crossover trial has confirmed the previous observations [46]. Intriguingly, during the short steroid trial, a paradoxical effect on the proliferative response to mitogen stimulation was documented, differently to what expected on the basis of the drug-induced immune suppression [7]. This finding would potentially imply a direct effect of betamethasone on the intimate altered pathogenetic mechanism in A-T [7].

However, as for the intimate mechanism implicated in such beneficial effect of the betamethasone, no conclusive information is available.

Peripheral effects of glucocorticoids

Most of the biologic effects of glucocorticoids (GCs) have been studied in white blood cells and, in particular, in lymphocytes. Different mechanisms of action have been described to explain GC therapeutic effects: the classical genomic mechanism of action mediated by the GC/GC receptor (GR) complex and non-genomic effects mediated by the cytoplasmic or membrane-bound GC/GR complex. The genomic mechanism may result in transrepression or transactivation [47] (Fig. 2). As for the transrepression (Fig. 2), GC/GR complex directly or indirectly interacts with transcription factors, including NF-KB, NF-AT, and AP-1 [48], thus resulting in the down-regulation of well-defined target genes [49]. Through this mechanism, GCs inhibit the synthesis of T-helper type 1 cytokines and, in particular, interleukin (IL)-2 [50], thus explaining the potent anti-inflammatory properties of these drugs. GCs also act, to a lesser extent, on the down-regulation of chemokine secretion and the expression of costimulatory molecules from immune and endothelial cells [51].

GCs seem to act downstream the TCR signal transduction pathway and, in particular, on the calcium signaling induced by inositol 1,4,5-triphosphate (IP3) [52]. It has been shown that low doses of dexamethasone on immature T cells (1–10 nM) induce the inhibition of Lck, thus altering calcium flux and the expression of IP3 receptor. In conclusion, these events eventually lead to the inhibition of the TCR-mediated signaling, thus explaining the inhibitory effect on cell activation [52]. By contrast, GCs seem to exert moderate effects on the survival, proliferation, and functionality of B cells [51].

Differently to this process, through a transactivation mechanism (Fig. 2), GC/GR complex directly interacts with molecular targets as GC-regulated genes. Following the interaction of GCs with GR, GRs migrate into the nucleus and, subsequently, bind to GC-responsive element (GRE), thus modulating the transcription of a variety of genes including GC-induced leucine zipper (GILZ) and GC-induced TNFR family-related (GITR) [53,54], which are the major biomarkers of GC-induced gene transcription. GILZ is a 137-amino-acid leucine zipper protein rapidly induced by GCs implicated in the modulation of activation-induced cell death [54,55], through inhibition of NF-kB translocation and activation. It is also able to regulate T lymphocyte activity, including T-cell survival [55,56].

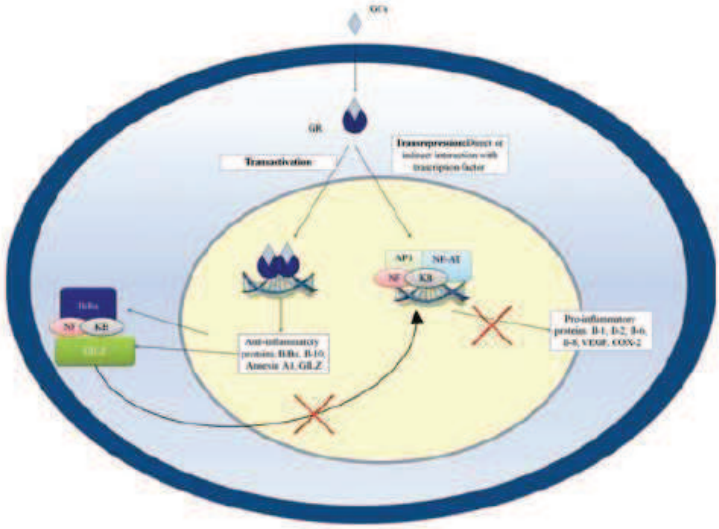

Figure 2 The classical, genomic mechanism of glucocorticoid action can be divided into two processes: 'transrepression' and 'transactivation'. Transrepression is the molecular mechanism by which GC/GR complex directly or indirectly interacts with transcription factors, including NF-KB, NF-AT, and AP-1, thus resulting in the down-regulation of well-defined target genes. In particular, through this mechanism, GCs inhibit the synthesis of T-helper type 1 cytokine. Transactivation is referred to the molecular mechanism by which GC/GR complex directly interacts with GC-regulated genes. GCs bind and induce GR activation, followed by the GR translocation to nucleus and subsequent binding to GC-responsive element (GRE), thus modulating the transcription of a variety of genes including GC-induced leucine zipper (GILZ) and GC-induced TNFR family–related (GITR) molecules, which are biomarkers of the GC-induced gene transcription.

GC effects on CNS

Neuroprotection

In the brain, the biologic function of GCs relies on their ability to access brain targets [57]. Data are available indicating that cortisol and corticosterone are capable of diffusing from plasma into the CNS by crossing the BBB and blood–cerebrospinal fluid barrier (BCSFB) [57]. However, the anatomical complexity of the brain enormously complicates the studies in the brain aimed at defining in detail gene targets of GC/GR complex [58]. Receptor binding and immunocytochemistry studies reveal that different regions of CNS do express GR, although the pattern of expression varies amongst the areas. GCs have been documented to exert neuroprotective, anticonvulsive, and anxiolytic properties [59]. GCs also have effects on the behavior mediated by non-genomic mechanisms [60]. Under physiological conditions, moderate increases in GCs have been shown to modulate cognition patterns, as well [59,61].

In several neurodegenerative disorders, inappropriate apoptosis seems to play an important pathogenic role. GCs exhibit a prominent role in the regulation of apoptosis, even though the modulatory effect occurs through opposite mechanisms under different circumstances. GCs enhance apoptosis in inflammatory and immune cells, whereas they seem to protect tissues in which the inflammation takes place from apoptotic death [62,63]. GCs promote apoptosis by inducing proteasome activity. GCs have been shown to protect neurons from apoptosis through a mechanism involving the cyclin-dependent kinase inhibitor p21Waf1/Cip1 molecule [62]. p21Waf1/Cip1 is upregulated and phosphorylated by GCs under the control of PI3-Akt kinase, eventually leading to the activation and cytoplasmic translocation of p21Waf1/Cip1 [62]. This modulatory pathway represents a novel antiapoptotic mechanism, which, in particular, exerts its effects on post-mitotic neurons, thus implying a direct role of p21Waf1/Cip1 as a molecular target of GC-induced neuroprotection [62]. Caspase-3 participates in the process, as well, being inhibited by p21Waf1/Cip1 [62].

GCs also act on neurological functions by upregulating HSP27 in the cerebellum [64]. This molecule belongs to the family of HSPs, which are small molecular weight proteins acting as molecular chaperones involved in the regulation of actin polymerization, thermotolerance, and cell growth [65]. HSPs have a protective function after exposure to several cellular insults, such as ROS. Synthesis of such proteins is

induced by GCs [64], and the resistance of the brain to stress-like elevations in corticosterone levels seems mediated by this mechanism [64]. The cerebellum is the principal site of induction of HSPs, and in particular HSP27, which seems to have a unique role in adapting neurons [66].

In conclusion, GCs act as inducers of protective systems against several insults in the brain. Several lines of evidence support the hypothesis that such mechanisms might have some site-directed specificity in the brain.

Synaptic plasticity

Plasticity refers to the ability of the CNS to reorganize itself over the time [67]. The main mechanism includes functional changes or alterations in the number or location of synapses between neurons [68]. Under physiological conditions, moderate increases in GCs have been shown to modulate synaptic plasticity and cognition patterns [69]. GRs exert their effects at the membrane, where they modulate the conductance of specific ion channels, which, in turn, modulate neuronal excitability [70]. GC injections in mice cause a dose-dependent effect on dendritic spine dynamics, increasing spine turnover within brain cortex [71]. Accordingly, a reduction in endogenous GC activity causes a substantial reduction in spine turnover rates [71]. However, chronic GC excess leads to abnormal loss of stable connections that were established early in life [71].

Synaptic plasticity requires brain-derived neurotrophic factor (BDNF) secretion and TrkB activation [72]. Trk receptors are involved in neuronal survival and differentiation [73,74]. Acute administration of dexamethasone promotes TrkB activation in the CNS *in vivo*, the effect not being mediated by BDNF. The activation of Trk receptors by GCs requires a genomic action of GR [75]. Also in the neurons, in addition to the genomic mechanism, GRs may also act in extranuclear areas, as the dendritic spine and axon terminal to induce the spinogenesis, or to modulate locally the synaptic transmission.

In a recent study, through functional magnetic imaging, an increase in the activation in relevant cortical areas has been shown to be coupled to changes in the motor performance in A-T patients treated with betamethasone [76]. This observation suggests that in patients with A-T, steroid treatment could improve motor performance facilitating cortical compensatory mechanisms. Changes in clusters of activation in response to motor tasks or sensory stimuli, related to the improvement in motor performance, have also been described during the rehabilitation of patients with different brain injuries, such as focal resection of the cortex or hemispherectomy [77] or stroke [68]. This ability to promote brain remodeling, reducing the impact of disability, has been related to the neuronal plasticity [68].

Conclusion

Currently, there is no effective treatment for A-T. Recent studies reported on the improvement of neurological signs during short-term treatment with oral betamethasone [6,7,45]. As for the mechanism underlying this effect of corticosteroids on neurological symptoms in A-T, no definitive explanation is currently available.

In this review, a possible mechanism implicated in the beneficial effect of steroids has been discussed on the basis of the pathogenesis, in particular, the GC effects on the suppression of inflammation or as an antioxidant through the activation of several biochemical pathways.

In several neurodegenerative disorders, and in particular in A-T, an inappropriate apoptosis seems to play an important pathogenic role. Studies show that GCs are able to enhance apoptosis in inflammatory and immune cells, whereas, at the same, time they seem to protect tissues in which the inflammation takes place [62,63]. GCs have been shown to protect post-mitotic neurons from apoptosis through a mechanism involving the cyclin-dependent kinase inhibitor p21Waf1/Cip1 molecule [62]. Of note, ATM signaling appears to function predominantly in immature, recently post-mitotic neurons [24].

Even though steroids are not curative and cannot be proposed for long-term therapies due to the side effects, it is noteworthy that betamethasone studies highlighted that during the clinical course of the disease, there is a phase when neurological impairment may be rescued at some extent. This observation will open novel therapeutic strategies.

Disclosure of conflict of interest

The authors declare no financial or other conflict of interests.

References

1. Crawford TO. Ataxia telangiectasia. *Semin Pediatr Neurol* 1998; **5:** 287–294.
2. Frappart PO, McKinnon PJ. Ataxia-Telangiectasia and related diseases. *Neuromolecular Med* 2006; **8:** 495–511.
3. Chun HH, Gatti RA. Ataxia-telangiectasia, an evolving phenotype. *DNA Repair* 2004; **3:** 1187–1196.

4. Aloj G, Giardino G, Valentino L, *et al*. Severe combined immunodeficiencies: new and old scenarios. *Int Rev Immunol* 2012; **31:** 43–65.
5. Gatti RA. Ataxia-Telangiectasia. In: Pagon RA, Bird TD, Dolan CR, Stephens K, eds. *GeneReviews™*. Seattle (WA): U.S. National Library of Medicine, 2010: 1993–1999.
6. Broccoletti T, Del Giudice E, Amorosi S, *et al*. Steroid-induced improvement of neurological signs in ataxia-telangiectasia patients. *Eur J Neurol* 2008; **15:** 223–228.
7. Broccoletti T, Del Giudice E, Cirillo E, *et al*. Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia. *Eur J Neurol* 2011; **18:** 564–570.
8. Russo I, Cosentino C, Del Giudice E, *et al*. In ataxia-telangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. *Eur J Neurol* 2009; **16:** 755–759.
9. Ball LG, Xiao W. Molecular basis of ataxia telangiectasia and related diseases. *Acta Pharmacol Sin* 2005; **26:** 897–907.
10. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev* 2001; **11:** 71–77.
11. Su TT. Cellular responses to DNA damage: one signal, multiple choices. *Annu Rev Genet* 2006; **40:** 187–208.
12. Caldecott KW. DNA single-strand breaks and neurodegeneration. *DNA Repair (Amst)* 2004; **3:** 875–882.
13. Guo Z, Deshpande R, Paull TT. ATM activation in the presence of oxidative stress. *Cell Cycle* 2010; **9:** 4805–4811.
14. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003; **421:** 499–506.
15. Valentin-Vega YA, Maclean KH, Tait-Mulder J, *et al*. Mitochondrial dysfunction in ataxia-telangiectasia. *Blood* 2012; **119:** 1490–1500.
16. Karbowski M, Neutzner A. Neurodegeneration as a consequence of failed mitochondrial maintenance. *Acta Neuropathol* 2012; **123:** 157–171.
17. Schapira AH. Mitochondrial diseases. *Lancet* 2012; **379:** 1825–1834.
18. Weissman L, de Souza-Pinto NC, Stevnsner T, Bohr VA. DNA repair, mitochondria, and neurodegeneration. *Neurosci* 2007; **145:** 1318–1329.
19. Barzilai A, Biton S, Shilol Y. The role of the DNA damage response in neuronal development, organization and maintenance. *DNA Repair* 2008; **7:** 1010–1027.
20. Callegari AJ, Kelly TJ. Shedding light on the DNA damage checkpoint. *Cell Cycle* 2007; **6:** 660–666.
21. Eyfjord JE, Bodvarsdottir SK. Genomic instability and cancer: networks involved in response to DNA damage. *Mutat Res* 2005; **592:** 18–28.
22. Lee Y, McKinnon PJ. Responding to DNA double strand breaks in the nervous system. *Neuroscience* 2007; **145:** 1365–1375.
23. Katyal S, McKinnon PJ. DNA repair deficiency and neurodegeneration. *Cell Cycle* 2007; **6:** 2360–2365.
24. Biton S, Gropp M, Itsykson P, *et al*. ATM-mediated response to DNA double strand breaks in human neurons derived from stem cells. *DNA Repair* 2007; **6:** 128–134.
25. Lavin MF, Gueven N, Bottle S, Gatti RA. Current and potential therapeutic strategies for the treatment of ataxia-telangiectasia. *Br Med Bull* 2007; **81–82:** 129–147.
26. Gatti RA, Becker-Catania S, Chun HH, *et al*. The pathogenesis of ataxia-telangiectasia. Learning from a Rosetta Stone. *Clin Rev Allergy Immunol* 2001; **20:** 87–108.
27. Ito K, Takubo K, Arai F, *et al*. Regulation of reactive oxygen species by Atm is essential for proper response to DNA double-strand breaks in lymphocytes. *J Immunol* 2007; **178:** 1613–1623.
28. Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. ATM activation by oxidative stress. *Science* 2010; **330:** 517–521.
29. Ceccatelli S, Tamm C, Zhang Q, Chen M. Mechanisms and modulation of neural cell damage induced by oxidative stress. *Physiol Behav* 2007; **92:** 87–92.
30. Brozmanov J, Duds A, Henriques JA. Repair of oxidative DNA damage an important factor reducing cancer risk. *Neoplasma* 2001; **48:** 85–93.
31. Dianov GL, Souza-Pinto N, Nyaga SG, Thybo T, Stevnsner T, Bohr VA. Base excision repair in nuclear and mitochondrial DNA. *Prog Nucleic Acid Res Mol Biol* 2001; **68:** 285–297.
32. Barzilai A, Rotman G, Shiloh Y. ATM deficiency and oxidative stress: a new dimension of defective response to DNA damage. *DNA Repair* 2002; **1:** 3–25.
33. Kultz D. Molecular and evolutionary basis of the cellular stress response. *Annu Rev Physiol* 2005; **67:** 225–257.
34. Kamsler A, Daily D, Hochman A, *et al*. Increased oxidative stress in Ataxia Telangiectasia evidenced by alternations in redox state of brains from Atm-deficient mice. *Cancer Res* 2001; **61:** 1849–1854.
35. Meredith MJ, Dodson ML. Impaired glutathione biosynthesis in cultured human ataxia-telangiectasia cells. *Cancer Res* 1987; **47:** 4576–4581.
36. Dean SW. Some aspects of glutathione metabolism in ataxia-telangiectasia fibroblasts. *Int J Radiat Biol Relat Stud Phys Chem Med* 1987; **52:** 43–48.
37. Dean SW, Sykes HR, Cole J, Jaspers NG, Linssen P, Verkerk A. Re: impaired glutathione biosynthesis in cultured ataxia-telangiectasia cells. *Cancer Res* 1988; **48:** 5374–5376.
38. Reichenbach J, Schubert R, Schindler D, Müller K, Böhles H, Zielen S. Elevated oxidative stress in patients with ataxia telangiectasia. *Antioxid Redox Signal* 2002; **4:** 465–469.
39. Stern N, Hochman A, Zemach N, *et al*. Accumulation of DNA damage and reduced levels of nicotine adenine dinucleotide in the brains of ATM-deficient mice. *J Biol Chem* 2002; **277:** 602–608.
40. Peter Y, Rotman G, Lotem J, Elson A, Shiloh Y, Groner Y. Elevated Cu/Zn-SOD exacerbates radiation sensitivity and hematopoietic abnormalities of ATM-deficient mice. *EMBO J* 2001; **20:** 1538–1546.
41. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J* 2011; **30:** 546–555.
42. Guo J, Rahman M, Cheng L, Zhang S, Tvinnereim A, Su DM. Morphogenesis and maintenance of the 3D thymic medulla and prevention of nude skin phenotype require FoxN1 in pre- and post-natal K14 epithelium. *J Mol Med* 2011; **89:** 263–277.
43. Jain M, Brenner DA, Cui L, *et al*. Glucose-6-phosphate dehydrogenase modulates cytosolic redox status and contractile phenotype in adult cardiomyocytes. *Circ Res* 2003; **93:** e9–e16.
44. Tuttle S, Stamato T, Perez ML, Biaglow J. Glucose-6-phosphate dehydrogenase and the oxidative pentose

phosphate cycle protect cells against apoptosis induced by low doses of ionizing radiation. *Radiat Res* 2000; **153:** 781–787.
45. Buoni S, Zanolli R, Sorrentino L, Fois A. Betamethasone and improvement of neurological symptoms in Ataxia-Telangiectasia. *Arch Neurol* 2006; **63:** 1479–1482.
46. Zannolli R, Sabrina B, Betti G, *et al.* A randomized trial of oral betamethasone to reduce ataxia symptoms in ataxia telangiectasia. *Mov Disord* 2012; **27:** 1312–1316.
47. Ehrchen J, Steinmuller L, Barczyk K, *et al.* Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes. *Blood* 2007; **109:** 1265–1274.
48. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 1995; **270:** 286–290.
49. Reily MM, Pantoja C, Hu X, Chinenov Y, Rogatsky I. The GRIP1:IRF3 interaction as a target for glucocorticoid receptor-mediated immunosuppression. *EMBO J* 2006; **25:** 108–117.
50. Northrop JP, Crabtree GR, Mattila PS. Negative regulation of interleukin 2 transcription by the glucocorticoid receptor. *J Exp Med* 1992; **175:** 1235–1245.
51. Zen M, Canova M, Campana C, *et al.* The kaleidoscope of glucocorticoid effects on immune system. *Autoimmun Rev* 2011; **10:** 305–310.
52. Harr MW, Rong Y, Bootman MD, Llewelyn Roderick H, Distelhorst CW. Glucocorticoid-mediated inhibition of Lck modulates the pattern of T cell receptor-induced calcium signals by down-regulating inositol 1, 4, 5-trisphosphate receptors. *J Biol Chem* 2009; **284:** 31860–31871.
53. Cannarile L, Zollo O, D'Adamio F, *et al.* Cloning, chromosomal assignment and tissue distribution of human GILZ, a glucocorticoid hormone-induced gene. *Cell Death Differ* 2001; **8:** 201–203.
54. Riccardi C, Cifone MG, Migliorati G. Glucocorticoid hormone induced modulation of gene expression and regulation of T-cell death: role of GITR and GILZ, two dexamethasone-induced genes. *Cell Death Differ* 2001; **6:** 1182–1189.
55. D'Adamio F, Zollo O, Moraca R, *et al.* A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* 1997; **7:** 803–812.
56. Riccardi C, Bruscoli S, Migliorati G. Molecular mechanisms of immunomodulatory activity of glucocorticoids. *Pharmacol Res* 2002; **45:** 361–368.
57. Mason BL, Pariante CM, Jamel S, Thomas SA. Central nervous system (CNS) delivery of glucocorticoids is fine-tuned by saturable transporters at the blood-CNS barriers and nonbarrier regions. *Endocrinology* 2010; **151:** 5294–5305.
58. Datson NA, Morsink MC, Meijer OC, de Kloet ER. Central corticosteroid actions: search for gene targets. *Eur J Pharmacol* 2008; **583:** 272–289.
59. McEwen BS. Stress and the aging hippocampus. *Front Neuroendocrinol* 1999; **20:** 49–70.
60. Evanson NK, Herman JP, Sakai RR, Krause EG. Nongenomic actions of adrenal steroids in the central nervous system. *J Neuroendocrinol* 2010; **22:** 846–861.
61. McEwen BS. Plasticity of the hippocampus: adaptation to chronic stress and allostatic load. *Ann N Y Acad Sci* 2001; **933:** 265–277.
62. Harms C, Albrecht K, Harms U, *et al.* Phosphatidylinositol 3-Akt-kinase-dependent phosphorylation of p21(Waf1/Cip1) as a novel mechanism of neuroprotection by glucocorticoids. *J Neurosci* 2007; **27:** 4562–4571.
63. Amsterdam A, Tajima K, Sasson R. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. *Biochem Pharmacol* 2002; **64:** 843–850.
64. Barr CS, Dokas LA. Glucocorticoids regulate the synthesis of HSP27 in rat brain slices. *Brain Res* 1999; **847:** 9–17.
65. Jakob U, Gaestel M, Engel K, Buchner J. Small heat shock proteins are molecular chaperones. *J Biol Chem* 1993; **268:** 1517–1520.
66. Satoh JI, Kim SU. Differential expression of heat shock protein HSP27 in human neurons and glial cells in culture. *J Neurosci Res* 1995; **41:** 805–818.
67. Johnston MV. Clinical disorders of brain plasticity. *Brain Dev* 2004; **26:** 73–80.
68. Johnston MV. Plasticity in the developing brain: implications for rehabilitation. *Dev Disabil Res Rev* 2009; **15:** 94–101.
69. Komatsuzaki Y, Murakami G, Tsurugizawa T, *et al.* Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus. *Biochem Biophys Res Commun* 2005; **335:** 1002–1007.
70. Prager EM, Johnson LR. Stress at the synapse: signal transduction mechanisms of adrenal steroids at neuronal membranes. *Sci Signal* 2009; **2:** re5.
71. Liston C, Gan WB. Glucocorticoids are critical regulators of dendritic spine development and plasticity *in vivo*. *PNAS* 2011; **108:** 16074–16079.
72. Fritsch B, Reis J, Martinowich K, *et al.* Direct current stimulation promotes BDNF-dependent synaptic plasticity: potential implications for motor learning. *Neuron* 2010; **66:** 198–204.
73. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* 2003; **72:** 609–642.
74. Toscano E, della Casa R, Mardy S, *et al.* Multisystem involvement in congenital insensitivity to pain with anhidrosis (CIPA), a nerve growth factor receptor(Trk A)-related disorder. *Neuropediatrics* 2000; **31:** 39–41.
75. Jeanneteau F, Garabedian MJ, Chao MV. Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *PNAS* 2008; **105:** 4862–4867.
76. Quarantelli M, Giardino G, Prinster A, *et al.* Steroid treatment in Ataxia-Telangiectasia induces alterations of functional magnetic resonance imaging during prono-supination task. *Eur J Paediatr Neurol* 2012; http://dx.doi.org/10.1016/j.ejpn.2012.06.002 [Epub ahead of print].
77. de Bode S, Mathern GW, Bookheimer S, Dobkin B. Locomotor training remodels fMRI sensorimotor cortical activations in children after cerebral hemispherectomy. *Neurorehabil Neural Repair* 2007; **21:** 497–508.

3.2. Autoimmunity and PIDs: a focus on APECED

Tolerance represents a state of immunologic non-responsiveness in the presence of a particular antigen. In this context, T-cell tolerance is crucial for the generation of a proper T-cell repertoire, able to respond to a huge number of foreign antigens, but preventing autoimmune reactions. The generation of appropriate self-tolerant the T-cell repertoire is exerted at two levels: (1) central tolerance (development and selection of T-cells in the thymus) and (2) peripheral tolerance (deletion, anergy of mature T-cells in lymphoid and non-lymphoid organs) (221). The intrathymic central tolerance is the mechanism by which T cells binding self-antigenswith high avidity, potentially autoreactive lymphocytes, are eliminated through the negative selectionprocess (221, 222, 223, 224). Autoreactive lymphocytes that have escaped negative selection are deleted in the periphery through the mechanisms of the peripheral tolerance (221).

Within the thymicmedulla negative selection ismediated bymTECs andmedullary DCs (mDCs).Mature mTECs display a peculiar phenotype, characterized by the high surface expression of CD40, CD80, CD86, andMHC class II, which favors an efficient thymocyte deletion. The prominent process in inducing the negative selection is represented by the broad expression of tissue-specific antigens (TSA).This high expression by mTECs of genes encoding TSA, normally found in the periphery, is driven by the transcription factor autoimmune regulator (AIRE) (222, 223, 224, 225). These TSA include, among other tissues, proteins restricted to the pancreas, stomach, eye, salivary gland, muscle, and thyroid (226, 227, 228, 229). However, AIRE doesn't regulate the expression of all TSA genes by mTECs. Surprisingly, only 1–2% of mTECs express a given TSA, making it very difficult to understand how a low number of mTECs is able to delete a so huge number of clonotypes (228). It has been

hypothesized that during a 4- to 5-day period the high motility of thymocytes allows them to make a huge number of interactions with mTECs (230). mDCs have a similar role, even though they acquire TSA mostly through the uptake of apoptotic mTECs. Alternatively, they migrate from the periphery into the thymus (231).

Some of the thymocytes that recognize self-MHC-peptide complexes with high affinity express Foxp3 and mature as Tregs, which are able to suppress autoreactive T cells in the periphery (232, 233). To mature into Tregs, the precursor cell must bind self-antigens with high avidity. In the mouse model, it has been shown that strong and not weak interactions between TCR and agonist peptide lead to the development of $Foxp3^+$ Tregs with regulatory properties (234, 235). However, there is evidence suggesting

the involvement of additional factors inthenatural differentiation of Tregs (236). Of note, two mechanisms to suppress self-reactive thymocytes have been proposed: triggering of co-stimulatory molecules and the activation of Fas signaling pathway. In particular, it has been shown that the co-stimulatory molecules such as CD40, CD80, and CD86, which are expressed by mTECs and mDCs, play a pivotal role in clonal deletion (237, 238). Indeed, exposure of DP thymocytes to anti-TCR monoclonal antibody (mAb) fails to induce thymocytes death, but not in the presence of APCs (239). In the presence of APCs expressing B7 molecules, a crucial co-stimulatory factor, which interacts with thymocyte CD28 receptor, DP thymocytes undergo apoptosis using anti-TCR and anti-CD28 mAb *in vitro*. Furthermore, it has been observed in SP thymocyte cultures that the exposure to high concentration of anti-TCR mAb is able to induce apoptosis through a high-level TCR signaling in the absence of co-stimulation (240). This effect is abolished in Fas-lpr/lpr cells (241), carrying

homozygous mutation of Fas, a member of the tumor necrosis factor receptor superfamily. Thus, the strong TCR ligation leads to apoptosis in a Fas-dependent manner in the absence of costimulatory signals (241). On the contrary, moderate-avidity TCR binding requires interactions with co-stimulatory molecules to induce apoptosis and, therefore, under these circumstances, cell death is induced by a Fas-independent mechanism.

The alteration of this orchestrated process leads to autoimmune diseases. Particularly, the prototype of a genetically determined failure of central tolerance leading to autoimmunity is APECED, caused by mutations in AIRE gene. APECED is a rare disease (OMIM 240300) (242) with a complex clinical phenotype discovered over decades (243). Patients with APECED have a highly variable pattern of destructive autoimmune reactions toward different endocrine and nonendocrine organs mainly involving parathyroid glands, adrenal cortex, gonads, pancreatic beta cells, gastric parietal cells, and thyroid gland. Moreover, ectodermal abnormalities are frequently present. The main ectodermal manifestations in APECED are dental enamel hypoplasia, pitted nail dystrophy, and alopecia. Keratopathy, vitiligo, and calcification of the tympanic membranes can also be described. Furthermore, autoimmunity in APECED may involve the gastrointestinal system, leading to autoimmune gastritis, malabsorption, and autoimmune hepatitis (244). The classical clinical diagnosis is primarily based on the presence of two of the three most common clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison disease (AD) (245).The presence of only one component is sufficient for the diagnosis if a sibling is affected (246). Autoantibodies for type 1 IFN (IFN-ω and IFN-

α) may be considered as a specific and sensitive diagnostic tool for APECED (247). Of note, the APECED varies in the severity and number of disease components.

So far, over 60 different mutations of the AIRE gene have been documented in APECED patients (248). Some different mutations have been found to be peculiar of specific geographic areas.

However, the great variability of APECED phenotype implies the participation of several disease-modifying genes and environmental factors to the disease phenotypic expression. In the near future, total exome sequencing could be a good perspective from which to analyze genetic variations involved in inheritance and clinical expression of autoimmune diseases.

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Genetic Basis of Altered Central Tolerance and Autoimmune Diseases: A Lesson from AIRE Mutations

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The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors. Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The immune system becomes tolerant to self-antigens through the two main processes, central and peripheral tolerance. Central tolerance takes place within the thymus and represents the mechanism by which T cells binding with high avidity self-antigens, which are potentially autoreactive, are eliminated through so-called negative selection. This process is mostly mediated by medullary thymic epithelia cells (mTECs) and medullary dendritic cells (DCs). A remarkable event in the process is the expression of tissue-specific antigens (TSA) by mTECs driven by the transcription factor autoimmune regulator (AIRE). Mutations in this gene result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), a rare autosomal recessive disease (OMIM 240300). Thus far, this syndrome is the paradigm of a genetically determined failure of central tolerance and autoimmunty. Patients with APECED have a variable pattern of autoimmune reactions, involving different endocrine and nonendocrine organs. However, although APECED is a monogenic disorder, it is characterized by a wide variability of the clinical expression, thus implying a further role for disease-modifying genes and environmental factors in the pathogenesis. Studies on this polyreactive autoimmune syndrome contributed enormously to unraveling several issues of the molecular basis of autoimmunity. This review focuses on the developmental, functional, and molecular events governing central tolerance and on the clinical implication of its failure.

Keywords AIRE, autoimmune diseases, autoimmunity, central tolerance, susceptibility genes

Recent evidence suggests that systemic autoimmunity and immunodeficiency can be strictly linked. One of the mechanisms is related to the decreased ability of immune system to clear the infections in patients with immunodeficiencies, which cause perpetual immune-system activation and, eventually, autoimmunity. Systemic autoimmunity is due to combined effect of multiple genetic variations, infections, and immunoregulatory factors that, along with a predominant autoimmune phenotype, may also lead to an increased susceptibility to infections. In the light of this novel point of view, the overlap of clinical manifestations suggests that immunodeficiency should be considered in the presence of autoimmunity and vice versa [1].

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Growing evidence has been accumulated indicating that autoimmune phenomena occur in patients suffering from primary immunodeficiencies (PID), and the molecular and cellular mechanisms that interconnect these conditions begin to be elucidated. The study of rare single-gene disorders associated with significant autoimmunity certainly and greatly contributed to the overall comprehension of the pathophysiology of the complex and intimate mechanisms underlying autoimmune disorders [2].

In this regard, monogenic autoimmune diseases, such as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), immunodysregulation, polyendocrinopathy and enteropathy X-linked (IPEX), autoimmune lymphoproliferative syndrome (ALPS), and interleukin (IL) 2 receptor α-chain (IL-2RA, CD25) deficiency, offer a unique model to unravel many aspects of the development, homeostasis, and function of the immune system as well as of the balance between autoimmunity and immunodeficiency. Under these conditions, central or peripheral tolerance, which normally prevents the survival, expansion, and activation of autoreactive T cells, thus protecting the system from autoimmune diseases, are impaired.

As a paradigm of abnormal peripheral tolerance, ALPS is a disorder characterized by nonmalignant lymphoproliferation, increased risk of lymphoma, and autoimmunity, often manifesting as multilineage cytopenias [3, 4]. The most common genetic alterations are heterozygous germline mutations in the gene encoding the TNF receptor-family member Fas (CD 95, Apo-1) [5–9], somatic Fas mutations and mutations in the genes encoding Fas-ligand (*FASLG*), caspase 10 (*CASP10*) and caspase 8 (*CASP8*), and NRAS and KRAS [10, 11].

IPEX is a further example of failure of the peripheral tolerance, characterized by immune dysfunction, polyendocrinopathy, enteropathy, and X-linked inheritance [12, 13]. This syndrome is caused by mutations in the forkhead box P3 (*FOXP3*) gene located in the short arm of chromosome X (Xq11.23-Xq13.3). This gene consists of 11 exons, which encode a protein of 431 amino acids, and serves as a lineage specification factor of regulatory T lymphocytes (Tregs) [14–16]. FOXP3 plays a crucial role in the generation of Tregs. Tregs are a thymus-derived cell subset [17], which plays a central role in the regulation of immune responses to self-antigens, allergens, and commensal microbiota as well as immune responses to infectious agents and tumors. A defective Treg function is associated with autoimmunity, allergy, and immunodeficiency [18, 19]. In contrast, an increased Treg function has been associated with malignancies [20–23].

In a few IPEX-like cases, alterations of other molecules involved in the generation of Tregs, such as the IL-2RA, have been reported [24, 25].

Although ALPS, IPEX, and IL-2RA deficiency are examples of genetic alterations that greatly contributed to a better understanding of the role of peripheral tolerance mechanisms, most of the current knowledge on the pathogenesis of autoimmunity also arises from the discovery of genetic models of abnormal central tolerance. In this review, we focus our attention on the paradigmatic genetic alterations resulting in the failure of central tolerance.

THYMIC DEVELOPMENT AND INTRATHYMIC SELECTION

The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors [26–28] and T-cell repertoire selection, which promotes differentiation of thymocytes expressing T-cell receptors (TCR) with intermediate affinity and/or avidity for self-peptide-MHC complexes and allows intrathymic removal of thymocytes that express TCR with high

affinity for self-antigens. These two processes, respectively known as positive and negative selection, are required to ensure a high degree of self-tolerance.

On entering the thymus, immature thymocytes promote the differentiation of precursor thymic epithelial cells (pTECs) into cortical TECs (cTECs) and medullary TECs (mTECs), playing an important role in the formation of the thymic microenvironment [29–31]. The passage of thymocytes through discrete thymic microenvironments together with the interaction with different types of stromal cells and antigen presenting cells (APCs) has an important role in the survival and fate choices of developing T cells.

On the basis of the different immunophenotypic patterns, T-cell development can be divided into three subsequent steps: the $CD4^-CD8^-$ DN stage, the $CD4^+CD8^+$ double-positive (DP) stage, and the $CD4^-CD8^+$ or $CD4^+CD8^-$ single-positive (SP) stage (Figure 1A). The DN1 thymocytes are multipotent cells in that they may still differentiate into B, T, myeloid, natural killer (NK), and dendritic cells (DCs) [32–34]. The DN2 thymocytes lose the multilineage potential even though, under certain circumstances, they can still differentiate into NK cells, DCs, or macrophages [35, 36]. DN2 to DN3 transition requires the expression of a different array of genes, which allows full TCRβ gene rearrangement in thymocytes, which become competent to undergo β-selection [37, 38]. The induction of recombinase activating gene-1 (*RAG-1*) and

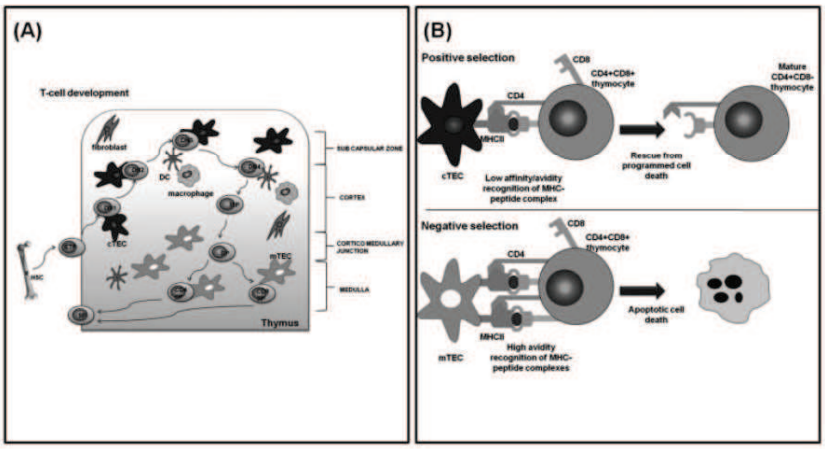

FIGURE 1. Thymic development and intrathymic selection. (A) The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors. The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. These steps are the entry of lymphoid progenitor cells into the thymus; the generation of $CD4^+CD8^+$ double positive (DP) thymocytes in the cortex; the positive selection of DP thymocytes in the cortex and the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete the thymocyte maturation; and, eventually, the export of mature T cells from the thymus. (B) Positive and negative selections are crucial for the creation of a T-cell repertoire able to respond to a huge number of foreign antigens, preserving, at the same time, the tolerance to self-antigens expressed in the various tissues. During positive selection (top panel) DP thymocytes, which do not get a rescue signal through TCR, are programmed to undergo "death by neglect" or apoptosis. Within the thymic medulla, negative selection (bottom panel) is mediated by mTECs and medullary DCs. The prominent process in inducing the negative selection is represented by the broad expression of tissue-specific antigens (TSA). T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, are eliminated through the negative selection process.

RAG-2, the upregulation of pre-Tα (*pTα*), and the rearrangement of TCRδ and γ also occur during the DN2/3 transition. T-cell precursors in this stage lose their capability to follow a non-T-cell fate choice [39]. The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage [40]. Pre-TCR signaling downregulates *CD25*, *pTα*, *RAG-1*, and *RAG-2*, leading to the DN4 stage. DN4 cells are fully committed to the $\alpha\beta$ T-cell lineage [41, 42].

After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point the cells become DP. Eventually, DP cells rearrange *TCRα* gene, leading to TCRα assembly into a TCR complex. This DP population, with an unselected repertoire, must undergo the positive selection [43, 44] to continue intrathymic development. DP thymocytes positively selected are induced to differentiate into SP cells and to migrate into medulla, where self-reactive thymocytes are deleted through negative selection.

Thereafter, SP thymocytes, with an appropriate TCR repertoire, leave the thymus as recent thymic emigrants (RTE), naïve cells expressing the CD62 ligand (CD62L), CD69, and the CD45RA isoform. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [45–47].

POSITIVE AND NEGATIVE SELECTION

Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. In this context, positive and negative selections are crucial for the creation of a T-cell repertoire able to respond to a huge number of foreign antigens, preserving, at the same time, the tolerance to self-antigens expressed in the various tissues [48].

DP thymocytes, in order to increase the ability to get through positive selection, rearrange the TCRα locus in a sequential manner, during a process referred to as receptor editing. After 3 days, DP thymocytes, which do not get a rescue signal through TCR, are programmed to undergo "death by neglect" or apoptosis (Figure 1B). Only about 5% of DP is able to bind a MHC ligand with mild avidity getting a signal that induces DP maturation to the $CD4^+$ $CD8^-$ or $CD4^-CD8^+$ SP stage [49–51] and triggers the expression of chemokines that direct the T cell through the subsequent maturation step within the thymic medulla [52, 53].

Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The intrathymic central tolerance is the mechanism by which T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, are eliminated through the negative selection process (Figure 1B) [48, 54–57]. Autoreactive lymphocytes that have escaped negative selection are deleted in the periphery through the mechanisms of the peripheral tolerance [48, 58].

Within the thymic medulla negative selection is mediated by mTECs and medullary DCs (mDCs). Mature mTECs display a peculiar phenotype, characterized by the high surface expression of CD40, CD80, CD86, and MHC class II, which favors an efficient thymocyte deletion. The prominent process in inducing the negative selection is represented by the broad expression of tissue-specific antigens (TSA) [59-62]. This high expression by mTECs of genes encoding TSA, normally found in the periphery, is driven by the transcription factor autoimmune regulator (AIRE) [59, 63–67]. These TSA include, among other tissues, proteins restricted to the pancreas, stomach, eye, salivary gland, muscle, and thyroid [59, 63, 68–70]. However, AIRE doesn't regulate the expression of all TSA genes by mTECs. Surprisingly, only 1–2% of mTECs express a given TSA, making it very difficult to understand how a low number of mTECs is able to delete a

so huge number of clonotypes [65, 67, 71]. It has been hypothesized that during a 4- to 5-day period the high motility of thymocytes allows them to make a huge number of interactions with mTECs [72, 73]. mDCs have a similar role, even though they acquire TSA mostly through the uptake of apoptotic mTECs. Alternatively, they migrate from the periphery into the thymus [74–76].

Some of the thymocytes that recognize self-MHC-peptide complexes with high affinity express Foxp3 and mature as Tregs, which are able to suppress autoreactive T cells in the periphery [64, 77–79]. To mature into Tregs, the precursor cell must bind self-antigens with high avidity [80]. In the mouse model, it has been shown that strong and not weak interactions between TCR and agonist peptide lead to the development of Foxp3$^+$ Tregs with regulatory properties [81–83]. However, there is evidence suggesting the involvement of additional factors in the natural differentiation of Tregs [84].

Of note, two mechanisms to suppress self-reactive thymocytes have been proposed: triggering of co-stimulatory molecules and the activation of Fas signaling pathway. In particular, it has been shown that the co-stimulatory molecules such as CD40, CD80, and CD86, which are expressed by mTECs and mDCs, play a pivotal role in clonal deletion [85, 86]. Indeed, exposure of DP thymocytes to anti-TCR monoclonal antibody (mAb) fails to induce thymocytes death, but not in the presence of APCs [87]. In the presence of APCs expressing B7 molecules, a crucial co-stimulatory factor, which interacts with thymocyte CD28 receptor, DP thymocytes undergo apoptosis using anti-TCR and anti-CD28 mAb in vitro [88]. Furthermore, it has been observed in SP thymocyte cultures that the exposure to high concentration of anti-TCR mAb is able to induce apoptosis through a high-level TCR signaling in the absence of co-stimulation [88]. This effect is abolished [89] in Fas-lpr/lpr cells [90], carrying homozygous mutation of Fas, a member of the tumor necrosis factor receptor superfamily. Thus, the strong TCR ligation leads to apoptosis in a Fas-dependent manner in the absence of co-stimulatory signals [89, 90]. On the contrary, moderate-avidity TCR binding requires interactions with co-stimulatory molecules to induce apoptosis and, therefore, under these circumstances, cell death is induced by a Fas-independent mechanism.

MOLECULAR BASIS OF THE CENTRAL TOLERANCE

Mouse and Human Models of Impaired Central Tolerance

To explain the huge number of tissue-specific proteins detected in the thymus, many studies have been till now conducted, in both mice and humans, to unravel the identity of peripheral self-antigen-producing cells within the thymus. Recent reports have shown that both in humans and mice, self-antigen expression in the thymus is restricted to mTECs [59, 91] and, in particular, to mTECs forming the Hassal corpuscle (HC). The molecular mechanisms governing self-antigen expression by mTECs of HC and the function of HC in the negative selection process remains to be clarified. Studies in human thymus show that mTECs in HC express CD30-L, a membrane-associated glycoprotein involved in T-cell signaling. Interestingly, CD30-deficient mice show a gross defect in negative but not positive T-cell selection [92].

Studies have shown that a reduced expression of a specific TSA by mTECs may be associated with the development of that specific autoimmune disorder. For example, susceptibility to type 1 diabetes (T1D) in humans has been strongly linked to polymorphisms in variable number of tandem repeats (VNTRs) within insulin promoter, which correlate with the level of mRNA expression within the thymus. A reduced thymic insulin expression may reduce the efficacy of clonal deletion promoting an increased escape of insulin-specific T cells. In addition, the variability of insulin expression levels in the thymus, when compared to the pancreas, also implies that insulin expression is regulated differently in these two organs [93]. Low expression of insulin in the

thymus of nonobese diabetic (NOD) mice has also been reported [94] and may play a role in diabetes susceptibility in this mouse strain. A point mutation in the *CHRNA1* gene, encoding the α-subunit of the muscle acetylcholine receptor has recently been described in a subset of patients with myasthenia gravis. These mutations are able to prevent the interferon (IFN) regulatory factor 8 binding to the *CHRNA1* gene, reducing its transcription in mTECs [95].

Negative selection of autoreactive thymocytes may also be impaired by alterations in TSA-specific mRNA processing. For example, in the mouse model of autoimmune encephalomyelitis, mRNA splicing deletes the expression of proteolipid protein peptide-specific thymocytes, thus leading to autoimmunity [96, 97].

Genetic alterations of thymocytes may also lead to autoreactive T-cell escape. It is well documented that the strength of signaling transduced by TCR upon binding of self-peptide MHC complex plays a key role in clonal deletion. For example, SKG mice, characterized by a point mutation in the C-terminus of the SH2-domain of ZAP-70, develop rheumatoid arthritis (RA)-like disease. Mutations in ZAP-70, in fact, result in reduced TCR signaling, thus limiting the deletion of the arthritogenic thymocytes [98]. A reduced thymic negative selection may also derive from a dysregulation of the pathways inducing apoptosis in the thymocytes. For example Nur77 is a pro-apoptotic molecule implicated in the thymocyte negative selection. In Nur77-deficient mice, autoreactive thymocytes are resistant to apoptosis, and reduced expression of Nur77 has been reported in a number of autoimmune disease [99].

Aberrant development of mTECs may also impact the efficacy of negative selection. For example, AIRE expression in the thymus requires cross-talk between developing thymocytes and stromal cells, and this process is dependent on an organized thymic microenvironment [100, 101]. A higher incidence of autoimmune phenomena, including autoimmune cytopenias [102, 103] autoimmune arthritis [104], and autoimmune endocrinopathies [105], can be observed in DiGeorge syndrome (DGS) [106, 107] in which abnormal thymic development may result in impaired expression of AIRE and, potentially, of other transcription factors that regulate expression of organ-specific antigens in the thymus resulting in defective central tolerance [100, 108]. However, it should be mentioned that in DGS autoimmunity may also be explained by exaggerated chronic inflammatory responses, "bystander" activation of autoreactive T cells and "molecular mimicry" [109]. Thymic abnormality in DGS may also impair the generation of Tregs. Evidence is available showing that patients with DGS have significantly lower Treg counts compared with healthy controls [110]. The decreased counts and proportions of Tregs in patients with developmental thymic hypoplasia suggest that the generation and maintenance of the FoxP3 Treg pool, at least in children, is directly related to thymic function rather than peripheral production [110].

Finally, a paradigmatic example of genetic alteration of central tolerance is the mutation of the *AIRE* gene. The *AIRE* gene maps in humans to chromosome 21q22.3, and was cloned in 1997 by two independent research groups [111, 112]. It consists of 14 exons spanning 11.9 kb of genomic DNA [113] and encodes a 58-kDa protein [111].

Three different isoforms of AIRE protein can be generated by alternative splicing. However, a unique combination of domains, common to transcriptional regulators and chromatin-binding proteins, has been identified. The N-terminal region shows a 6-helix structure with high similarity to a caspase-recruitment domain (CARD) [114]. This CARD domain is required for oligomerization of AIRE [115, 116]. Moreover, AIRE contains an Sp100, AIRE1, NucP41/75, DEAF1 (SAND) domain [117] important for AIRE transactivation capacity and subcellular localization [118]. The C-terminal region contains 2 plant homeodomain PHD- type zinc fingers [119]. Mutations in the PHD domains lead to a severe decrease of AIRE transcriptional activation capacity [120–122]. In particular, disease-causing missense mutations in PHD1 domain have

been shown to abolish E3 ligase activity [123]. Other structural features include a conserved nuclear localization signal and 4 LXXLL motifs or nuclear receptor interaction domains [124]. AIRE protein is mainly localized in the cell nucleus, within nuclear bodies distributed near to nuclear speckles [120], suggesting the involvement of AIRE in the regulation of gene transcription.

The precise molecular mechanisms underlying AIRE function remain to be clarified. The large number and chromosomal clustering [125] of the AIRE-regulated gene suggest that it does not act as a conventional DNA-binding transcription factor, but as a coactivator in a large transcriptional complex. Moreover, recent studies on human embryonic kidney 293T cells have demonstrated that AIRE interacts with a large set of binding partners [126]. The proteins could be divided into 4 major functional classes: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing [126]. The first protein reported to bind to AIRE was CREB-binding protein (CBP) [127]. CBP is important for transcription initiation and histone and nonhistone protein acetylation. Its interactions with AIRE may lead to promotion of gene transcription through histone acetylation and the recruitment of chromatin-transcription factors [115, 128, 129]. Other AIRE partners have been identified, such as DNA protein kinase (DNA-PK) and SP-RING domain protein inhibitor of activated STAT1 (PIAS1) (Figure 2) [130, 131]. DNA-PK deficiency in the stromal compartment leads to decreased TSA expression in mTECs, thus resulting in autoantibody production [122, 130]. AIRE also binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II, eventually targeting gene promoters and enhancing the transcription-elongation process [132]. The ability of AIRE's PHD1 finger domain to bind histone 3 molecules with unmethylated lysine at position 4, generally associated with repressed genes, indicates a possible epigenetic control of the AIRE target genes [133, 134]. It is plausible that AIRE recruitment of all its co-transcriptional partners to TSA gene promoters activates the expression of the large number of TSA in mTECs [135].

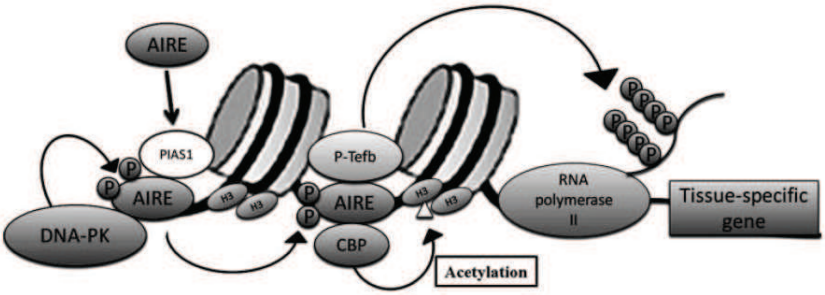

FIGURE 2. AIRE's PHD1 finger domain is preferentially recruited to histone 3 molecules with unmethylated lysine at position 4, indicating a possible epigenetic control of the AIRE target genes. On target gene regulatory regions, AIRE recruits a number of partners, which could be divided into 4 major functional classes: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing. AIRE binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II enhancing the transcription-elongation process. AIRE also interacts with CREB-binding protein (CBP), leading to promotion of gene transcription through histone acetylation and recruitment of chromatin-transcription factors. AIRE may also interact with the DNA-dependent protein kinase (DNA-PK) complex and protein inhibitor of activated STAT1 (PIAS1). DNA-PK phosphorylates AIRE and collaborates with AIRE in the formation of chromatin loops. PIAS1 is involved in the nuclear organization of chromatin.

AIRE has also been detected in peripheral lymphoid tissues, such as lymph nodes and spleen, and in other tissues, though at a lower degree than in thymic stromal cells. It is likely that in the periphery, AIRE also contributes to the process of immune tolerance by inducing TSA's gene expression [136–139].

The murine *Aire* gene has been mapped to chromosome 10, revealing a structural organization and sequence homology to its human ortholog [140, 141]. Animal models of $Aire^{-/-}$ have been an important tool in furthering our understanding of how *Aire* prevents autoimmunity. $Aire^{-/-}$ mice present several autoimmune manifestations, mononuclear infiltrates in multiple organs, and the presence of autoantibodies against several tissues [63, 116, 142]. Differently from the human counterpart, $Aire^{-/-}$ mice do not develop *Candida* infections [143].

Along with the central tolerance network, several other peripheral mechanisms are capable of contributing to the control and regulation of the immune system and, presumably, to the clinical expression of the disease. An additional mechanism involved in controlling the reactivity to self-antigens in the periphery is also represented by NK-cell activity. Studies show that resting or cytokine-induced NK cells are able to inhibit activation and/or proliferation of autoreactive clones, as well [144–147].

An example of the association of AIRE alterations with immunodeficiency and autoimmunity is Omenn syndrome [100, 148]. AIRE expression in the thymi of 2 Omenn syndrome patients and 1 $T^-B^-NK^+$ shows profound reduction of AIRE mRNA and protein compared to a normal control subject. Moreover, there was no detectable mRNA for the self-antigens insulin, cytochrome P450 1A2, or fatty acid-binding protein in the immunodeficient patients. The authors concluded that deficiency of AIRE expression occurs in severe immunodeficiencies characterized by abnormal T-cell development and suggested that in Omenn syndrome, the few residual T-cell clones that develop may escape negative selection and thereafter expand in the periphery, causing massive autoimmune reactions [100].

The Human Phenotype Associated with AIRE Gene Mutations: APECED

APECED is a rare disease (OMIM 240300) [111, 112] with a complex clinical phenotype discovered over decades [149]. Patients with APECED have a highly variable pattern of destructive autoimmune reactions toward different endocrine and nonendocrine organs mainly involving parathyroid glands, adrenal cortex, gonads, pancreatic beta cells, gastric parietal cells, and thyroid gland. Moreover, ectodermal abnormalities are frequently present. The main ectodermal manifestations in APECED are dental enamel hypoplasia, pitted nail dystrophy, and alopecia. Keratopathy, vitiligo, and calcification of the tympanic membranes can also be described. Furthermore, autoimmunity in APECED may involve the gastrointestinal system, leading to autoimmune gastritis, malabsorption, and autoimmune hepatitis [150]. The classical clinical diagnosis is primarily based on the presence of two of the three most common clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison disease (AD) [151]. The presence of only one component is sufficient for the diagnosis if a sibling is affected [152]. Autoantibodies for type 1 IFN (IFN-ω and IFN-α) may be considered as a specific and sensitive diagnostic tool for APECED [121, 153, 154]. The APECED varies in the severity and number of disease components. In most patients, CMC precedes the other immune disorders appearing by the age of 5 years, usually followed by CH and later by AD [155]. The complete triad develops in up to two-thirds of patients [151, 152]. As mentioned above, further clinical or latent autoimmune endocrine diseases may be associated [151]. The autoimmune manifestations most likely result from destruction of the target organ by cellular and antibody-mediated attack [152]. In particular, autoantibodies to parathyroid, adrenal glands, and type I IFN are hallmarks of APECED [121, 153]. The molecular

basis of the increased susceptibility to CMC in APECED patients is still poorly understood. Recently, a role of the autoantibodies against the Th17-related cytokines IL-22, IL-17A, and IL-17F has been described in the pathogenesis of the CMC [156, 157]. In particular, IL-17F and IL-22 secretion seems to be significantly decreased in response to *Candida albicans* in APECED patients [156]. Life expectancy depends on the severity of the disease. The overall mortality of patients with APESED is high, but it widely varies on the basis of the clinical spectrum. The most dangerous autoimmune manifestations are fulminant necrotizing hepatitis, severe malabsorption, and tubulointerstitial nephritis [158]. Suboptimal hormonal substitution or inadequate management of addisonian crisis may also increase the mortality risk [159]. Furthermore, patients with long-lasting oral candidiasis are at increased risk of esophageal squamous cell carcinoma [160].

Disease-targeted therapy is not currently available and the treatment mainly relies on hormone replacement and caring for clinical symptoms. So far, immunosuppressant therapy has been considered only for potentially fatal disease, such as hepatitis, nephritis, or severe malabsorption [161]. In our series of patients with childhood onset APECED, the clinical spectrum widely varies between patients suffering only occasional episodes of mild oral candidiasis and stable hypoparathyroidism and patients exhibiting complex phenotypes with life-threatening events [162, 163]. Most of them are well controlled with only hormonal replacement therapy. Experimental immunomodulatory treatment of *Aire* knockout mouse targeting T and B cells might open the perspective of a similar strategy also in humans [164, 165].

Although rare, APECED has been reported worldwide [150] with a wide variability in its incidence. The estimated prevalence in some genetically isolated populations is relatively higher (1:9000 in the Iranian Jews [166], 1:25000 in Finns [152, 167], and 1:14.400 in Sardinians [168]. It is also quite frequent in Norway [154] and Italy [162]. Even though the most frequent mode of inheritance is autosomal recessive, an Italian family with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy harbored a missense (G228W) mutation in the exon 6 in heterozygosity, indicating a dominant pattern of inheritance [169].

So far, over 60 different mutations of the *AIRE* gene have been documented in APECED patients [70]. Some different mutations have been found to be peculiar of specific areas. R257X is the most common mutation among Finnish and other European patients [170–172], 1094-1106del113 (or 967-979del13 bp) is the most common mutation in British [173], Irish [174], North America [175, 176], and Norwegian patients [154], and the Y85C mutation is more frequent among Iranian Jews [120]. In Italy APECED shows an increased prevalence in various regions, in particular in Sardinia, Apulia, and the Venetian area. Moreover, both in Sardinia and Apulia peculiar mutations of *AIRE* have been identified: the mutation R139X on exon 3 in Sardinia [177, 178] and the mutation W78R on exon 2 in Apulia [179]. In the Veneto region, *AIRE* mutations (R257X on exon 6 and 8) were different from the other Italian regions but similar to that identified in Finnish and Anglo-Saxon patients [180]. A typical mutation has been recently identified also in Sicily (R203X on exon 5) [181, 182]. The patients from Campania showed high frequency of mutations in the region of exon/intron 1 [162]. No AIRE gene mutations specific to Calabria have been found in patients with APECED. Only mutations similar to those found in patients from Apulia and Sicily were identified [183].

As mentioned above, although APECED is a monogenic disorder, it is characterized by a wide variability in clinical expression, each patient showing a different pattern of affected organs and autoantibody specificities. In the largest reported series of 91 Finnish patients, a wide variation of the clinical phenotype and course of APECED has been documented [150]. Later on, many other authors confirmed this phenotypic

heterogeneity among several populations [162, 167, 168, 171, 174, 176, 180, 184] but a precise genotype–phenotype correlation is still lacking. Of note, the clinical features vary not only among patients from different families but also among siblings carrying the same mutation [162]. Such variability strongly suggests that disease-modifying genes, environmental factors, as well as immune system dynamics may play a role in modulating clinical expression of the syndrome.

More recent analyses revealed effects of additional genetic loci, in particular the human leukocyte antigen (HLA) complex, on certain disease manifestations of APECED [70, 185]. Associations with specific HLA haplotypes have been found for trait components like alopecia, AD, and T1D in patients with APECED. These haplotypes are those associated with the common, non-APECED-related forms of the specific disorder. However, only a weak association has been observed between the HLA type and autoantibody specificities in APECED patients, suggesting that in APECED the HLA alleles do not have a strong influence on autoantibody formation [185]. To date, there are only few studies on the functionality of peripheral immunological tolerance mechanisms in patients with APECED. An impairment of $CD4^+CD25^+$ Tregs in adult APECED patients has been reported [186]. However, the reduction in circulating Tregs might also be secondary to the chronic fungal infection in these individuals.

Recently, several genetic, environmental, and molecular factors potentially implicated in the phenotypic variability of APECED were investigated in two siblings affected with APECED. They were characterized by an extremely different phenotypic expression despite an identical mutation of *AIRE* (IVS1 + 1G>C; IVS1 + 5delG mutation) [162]. In particular, the younger sister had a mild form of the syndrome, while the older male developed a severe phenotype exhibiting an accelerated phase involving parathyroid, thyroid, oral mucosa, skin, liver, adrenal glands, bowel, and stomach, culminating in a life-threatening posterior encephalopathy syndrome never described before in the context of APECED [163]. So far, there is no evidence that the severity of the disease is influenced by sex in that, unlike other autoimmune diseases, APECED affects in equal manner males and females. The sibs were compared for exposure to infectious triggers (rubella, Epstein Barr virus, cytomegalovirus, toxoplasma, varicella zoster virus, parvovirus B19, herpes simplex virus, and parainfluenza virus) [187, 188], autoantibodies profile, mechanisms of peripheral tolerance (Fas-induced apoptosis, number of $TCD4^+CD25^+$ regulatory cells, and NK activity) and HLA haplotype. The results suggested that differences in the exposure to common triggering infectious agents or functionality of mechanisms governing peripheral tolerance were not involved in the clinical variability between the two sibs. However, there is the possibility that the difference in the genetic pattern between the two siblings could be responsible for the high variability in the clinical course. In fact, for APECED, as for other mendelian disorders, the interplay between multiple genetic, epigenetic and environmental factors certainly play a role in phenotypic variability of APECED. As APECED represents a paradigmatic example of monogenic disease with a very heterogeneous clinical expression, other complex autoimmune diseases are characterized by a strong genetic inheritance although not related to a single gene mutation.

Autoimmune thyroid disease (AITD) is an example of complex interaction between genetic, epigenetic, and environmental factors. Epidemiological data, including family and twin studies, revealed a strong genetic susceptibility with a positive family in about half of the patients. Moreover, genetic anticipation, which is common in mendelian diseases, has been documented in cohorts of AITD families [189]. Taken together, these observations suggest that the inheritance of an "at-risk" genotype creates a susceptible background predisposing to development of AITD. However, AITD is characterized by an extreme variability in the clinical expression even

when familial inheritance is well documented. To unravel the intrinsic mechanisms of the genetic influence and the modulation of clinical expression in AITD, several genes, mainly involved in the cross-talk between APCs and T cells in the immunological synapse, have been studied. Main susceptibility genes are summarized in Table 1. These genes can be divided as follows: immunological synapse genes (HLA-DR, cytotoxic T-lymphocyte antigen-4 [CTLA4], lymphoid tyrosine phosphatise non-receptor [PTPN22], and B lymphocyte surface immunoreceptor [CD40]) (Figure 3), T-regulatory gene (FOXP3, IL-2RA), and thyroid-specific genes (thyroglobulin and TSH receptor [TSHR]).

Studies on all these genes have revealed that, although several polymorphic variations of all the cited genes have been identified and linked to AITD, most of them have only a weak effect on the increased genetic susceptibility to develop the disease. Familial clustering in complex autoimmune diseases does not necessarily mean that the disease is genetic in nature. Familial clustering can also be stochastic, or result from a combination of shared extrinsic and intrinsic factors. On the other hand, it is possible that in monogenic disease, an interplay between multiple genetic and molecular factors may be involved in modulating the clinical expression of the disease.

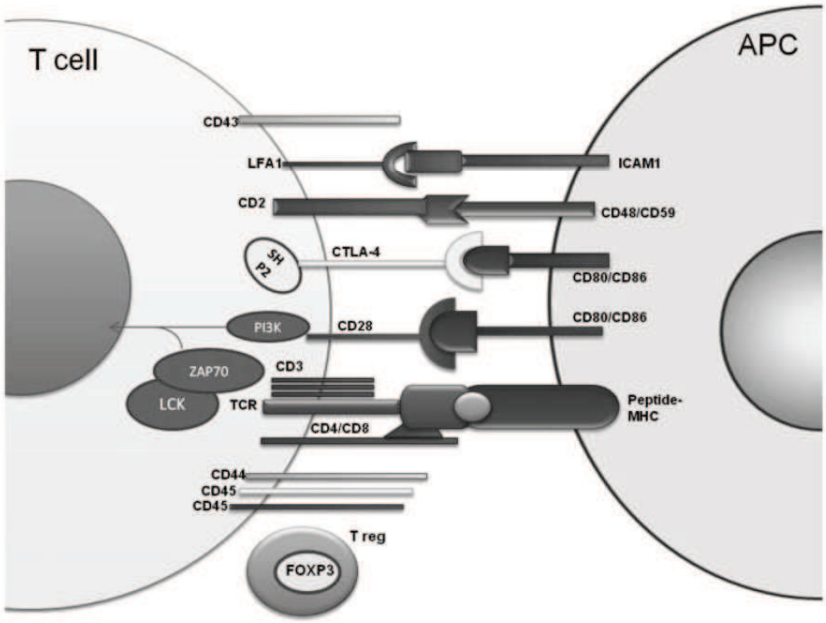

FIGURE 3. Immunological synapse. A summary view of the key ligand pairs and signaling molecules that are involved in T-cell recognition. CD80/CD86 is stimulatory peptide-MHC molecule; CD48/CD59, ICAM1, LFA1, CD2, CD28, CD3, CD4/CD8, and CD45 are activating/co-stimulatory molecules; CTLA-4 is an inhibitory molecule; and CD43 and CD44 do not contribute to signaling. The arrow indicates converging signals that lead to T-cell activation. APC, antigen-presenting cell; CD44/45, cell-surface glycoproteins; CTLA4, cytotoxic T-lymphocyte antigen 4; FOXP3, forkhead box P3; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; T reg, regulatory T-cell; ZAP70, ξ-chain-associated protein 70.

TABLE 1. Susceptibility genes for autoimmune thyroid disease (AIDT)

Candidate gene	Chromosome	Function
Human leukocyte antigen (HLA)-DR	6p21	Located on professional antigen-presenting cells (APCs) and involved in presenting peptides to lymphocytes
Cytotoxic T-lymphocyte associated protein 4 (CTLA4)	2q33	Negative regulation of T-lymphocyte activation
Protein tyrosine phosphatase non receptor 22 (PTPN22)	1p13	Lymphoid-specific intracellular phosphatase involved in regulating the T-cell receptor signaling pathways
B-lymphocyte surface immunoreceptor (CD 40)	20q	Expressed primarily on B cells and other APCs; important role in B-cell activation and antibody secretion
T-regulatory gene (FOXP3)	Xp11.23	Key gene for the differentiation of T cells into natural Treg cells
Interleukin 2 receptor α-chain (IL2RA)	10p15	Element of the high-affinity IL2 receptor, involved in IL2 signaling; present on many T-cell subsets; regulator of Treg cells
Thyroid-stimulating hormone receptor (TSHR)	14q31	G-protein-coupled receptor with a central role in controlling thyroid cell metabolism
Thyroglobulin (Tg)	8q24	Involved in the production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3)

CONCLUSIONS

Recent evidence indicates that systemic autoimmunity and immunodeficiency can be strictly linked. The discovery of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms enormously contributed to our understanding of the molecular basis of human autoimmune disorders, generally and appropriately considered as multifactorial diseases. The paradigm of the genetically determined alteration of central tolerance is related to mutations of the AIRE transcription factor. This disease gave a huge amount of information on the functional and molecular events that lead to the elimination of self-reactive T-cell clones within the thymus. The elucidation that TSA are broadly expressed by mTECs also helped in understanding the mechanism by which autoimmunity may also paradoxically be associated with an immunodeficiency status. However, the great variability of APECED phenotype implies the participation of several disease-modifying genes and environmental factors to the disease phenotypic expression. Since these elements are presumably pathogenetically relevant in inducing nonmonogenic autoimmune disorders, such as AITD, and to explain the autoimmune susceptible background, which is very impressive in certain families, studies of these monogenic models of disease may help unravel the pathogenesis of autoimmunity. In the near future, total exome sequencing could be a good perspective from which to analyze genetic variations involved in inheritance and clinical expression of autoimmune diseases.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Grammatikos A, Tsokos G. Immunodeficiency and autoimmunity: lessons from systemic lupus erythematosus. Trends Mol Med. 2012;18:101–108.

[2] Pessach IM, Notarangelo L. X-linked primary immunodeficiencies as a bridge to better understanding X-chromosome related autoimmunity. J Autoimmun. 2009;33:17–24.
[3] Madkaikar M, Mhattre S, Gupta M, Ghosh K. Advances in autoimmune lymphoproliferative syndromes. Eur J Haematol. 2011;87:1–9.
[4] De Berardinis P, Ombra MN, Buono C, Toraldo R, Vetrano F, Guardiola J. Long-term culture and T cell receptor analysis of T cell clones isolated from a patient with adenosine deaminase deficiency and type I diabetes. Clin Immunol Immunopathol. 1994;73:362–366.
[5] Sneller MC, Strans SE, Jaffe ES, et al. A novel lymphoproliferative/autoimmune syndrome resembling murine Ipr/gld disease. J Clin Invest. 1992;90:334–341.
[6] Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81:935–946.
[7] Puck JM, Rieux-Laucat F, Le Deist F. Autoimmune lymphoproliferative syndrome. In: Ochs H, Smith CIE, Puck JM, eds. Primary Immunodeficiency Diseases: A Molecular and Genetic Approach. New York: Oxford University Press; 2006: 326–341.
[8] Niemela JE, Lu L, Fleisher TA, et al. Somatic KRAS mutations associated with a human nonmalignant syndrome of autoimmunity and abnormal leukocyte homeostasis. Blood. 2011;117:2883–2886.
[9] DeFranco S, Bonissoni S, Cerutti F, et al. Defective function of Fas in patients with type 1 diabetes associated with other autoimmune diseases. Diabetes. 2001;50:483–488.
[10] Turbyville JC, Koneti Rao V. The autoimmune lymphoproliferative syndrome: a rare disorder providing clues about normal tolerance. Autoimmun Rev. 2010;9:488–493.
[11] Holzelova E, Vonarbourg C, Stolzenberg MC, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. N Engl J Med. 2004;351:1409–1418.
[12] Godfrey VL, Wilkinson JE, Russell LB. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. Am J Pathol. 1991;138:1379–1387.
[13] Bennett CL, Ochs HD. IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. Curr Opin Pediatr. 2001;13:533–538.
[14] Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet. 2002;39:537–545.
[15] Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27:20–21.
[16] Gambineri E, Perroni L, Passerini L, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent correlation between forkhead box protein 3 expression and disease severity. J Allergy Clin Immunol. 2008;122:1105–1112.
[17] Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057–1061.
[18] Bacchetta R, Gambineri E, Roncarolo MG. Role of regulatory T cells and FOXP3 in human diseases. J Allergy Clin Immunol. 2007;120:227–235.
[19] Romagnani S. Immunological tolerance and autoimmunity. Intern Emerg Med. 2006;1:187–196.
[20] Karube K, Ohshima K, Tsuchiya T, et al. Expression of FoxP3, a key molecule in CD4CD25 regulatory T cells, in adult T-cell leukaemia/lymphoma cells. Br J Haematol. 2004;126:81–84.
[21] Viguier M, Lemaître F, Verola O, et al. Foxp3 expressing CD4$^+$CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol. 2004;173:1444–1453.
[22] Berger CL, Tigelaar R, Cohen J, et al. Cutaneous T-cell lymphoma: malignant proliferation of T-regulatory cells. Blood. 2005;105:1640–1647.
[23] Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res. 2005;65:2457–2464.
[24] Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol. 2005;116:949–959.
[25] Roifman CM. Human IL-2 receptor alpha chain deficiency. Pediatr Res. 2000;48:6–11.
[26] Miller JF. Immunological function of the thymus. Lancet. 1961;2:748–749.
[27] Bleul CC, Boehm T. Chemokines define distinct microenvironments in the developing thymus. Eur J Immunol. 2000;30:3371–3379.
[28] Liu C, Ueno T, Kuse S, et al. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. Blood. 2005;105:31–39.
[29] Holländer GA, Wang B, Nichogiannopoulou A, et al. Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. Nature. 1995;373:350–353.

[30] van Ewijk W, Hollander G, Terhorst C, Wang B. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. Development. 2000;127:1583–1591.
[31] Klug DB, Carter C, Gimenez-Conti IB, Richie ER. Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. J Immunol. 2002;169:2842–2845.
[32] Shortman K, Wu L. Early T lymphocyte progenitors. Annu Rev Immunol. 1996;14:29–47.
[33] Wu CY, Warrier RR, Carvajal DM, et al. Biological function and distribution of human interleukin-12 receptor b chain. Eur J Immunol. 1996;246:345–350.
[34] Moore TA, Zlotnik A. T-cell lineage commitment and cytokine responses of thymic progenitors. Blood. 1995;86:1850–1860.
[35] Lee CK, Kim JK, Kim Y, et al. Generation of macrophages from early T progenitors in vitro. J Immunol. 2001;166:5964–5969.
[36] Schmitt TM, Ciofani M, Petrie HT, Zúñiga-Pflücker JC. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. J Exp Med. 2004;200:469–479.
[37] Talebian L, Li Z, Guo Y, et al. T-lymphoid, megakaryocyte, and granulocyte development are sensitive to decreases in CBFbeta dosage. Blood. 2007;109:11–21.
[38] Bender TP, Kremer CS, Kraus M, Buch T, Rajewsky K. Critical functions for c-Myb at three checkpoints during thymocyte development. Nat Immunol. 2004;5:721–729.
[39] Anderson G, Jenkinson WE, Jones T, et al. Establishment and functioning of intrathymic microenvironments. Immunol Rev. 2006;209:10–27.
[40] von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. Annu Rev Immunol. 1997;15:433–452.
[41] Zúñiga-Pflücker JC, Lenardo MJ. Regulation of thymocyte development from immature progenitors. Curr Opin Immunol. 1996;8:215–224.
[42] Fehling HJ, von Boehmer H. Early alpha beta T cell development in the thymus of normal and genetically altered mice. Curr Opin Immunol. 1997;9:263–275.
[43] Kisielow P, Teh HS, Blüthmann H, von Boehmer H. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature. 1988;335:730–733.
[44] Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. Annu Rev Immunol. 1995;13:93–126.
[45] Reichert RA, Weissman IL, Butcher EC. Phenotypic analysis of thymocytes that express homing receptors for peripheral lymph nodes. J Immunol. 1986;136:3521–3528.
[46] Bendelac A, Matzinger P, Seder RA, Paul WE, Schwartz RH. Activation events during thymic selection. J Exp Med. 1992;175:731–742.
[47] Ramsdell F, Jenkins M, Dinh Q, Fowlkes BJ. The majority of $CD4^+8^-$ thymocytes are functionally immature. J Immunol. 1991;147:1779–1785.
[48] Griesemer AD, Sorenson EC, Hardy MA. The role of the thymus in tolerance. Transplantation. 2010;90:465–474.
[49] Cheroutre H. Starting at the beginning: new perspectives on the biology of mucosal T cells. Annu Rev Immunol. 2004;22:217–246.
[50] Franki AS, Van Beneden K, Dewint P, et al. Lymphotoxin alpha 1 beta 2: a critical mediator in V alpha 14i NKT cell differentiation. Mol Immunol. 2005;42:413–417.
[51] Baldwin TA, Hogquist KA, Jameson SC. The fourth way? Harnessing aggressive tendencies in the thymus. J Immunol. 2004;173:6515–6520.
[52] Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids in vivo. J Immunol. 2002;169:1837–1843.
[53] Martin S, Bevan MJ. Antigen-specific and nonspecific deletion of immature cortical thymocytes caused by antigen injection. Eur J Immunol. 1997;27:2726–2736.
[54] Palmer E. Negative selection—clearing out the bad apples from the T-cell repertoire. Nat Rev Immunol. 2003;3:383–391.
[55] Hammerling GJ. Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen. Immunol Rev. 1991;122:47–67.
[56] McGargill MA, Derbinski JM, Hogquist KA. Receptor editing in developing T cells. Nat Immunol. 2000;1:336–341.
[57] Sakaguchi S. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol. 2005;6:345–352.
[58] Pignata C, Troncone R, Monaco G, et al. Impaired suppressor activity in children affected by coeliac disease. Gut. 1985;26:285–290.
[59] Derbinski J, Schulte A, Kyewski B, Klein L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol. 2001;2:1032–1039.

[60] Derbinski J, Gabler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J Exp Med. 2005;202:33–45.
[61] Gray DH, Seach N, Ueno T, et al. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood. 2006;108:3777–3785.
[62] Gray D, Abramson J, Benoist C, Mathis D. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J Exp Med. 2007;204:2521–2528.
[63] Anderson MS, Venezianí ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science. 2002;298:1395–1401.
[64] Coutinho A, Caramalho I, Seixas E, Demengeot J. Thymic commitment of regulatory T cells is a pathway of TCR-dependent selection that isolates repertoires undergoing positive or negative selection. Curr Top Microbiol Immunol. 2005;293:43–71.
[65] Derbinski J, Pinto S, Rösch S, Hexel K, Kyewski B. Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. Proc Natl Acad Sci U S A. 2008;105:657–662.
[66] Gabler J, Arnold J, Kyewski B. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. Eur J Immunol. 2007;37:3363–3372.
[67] Gillard GO, Farr AG. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. J Immunol. 2006;176:5815–5824.
[68] Kyewski B, Peterson P. Aire, master of many trades. Cell. 2010;140:24–26.
[69] Mathis D, Benoist C. A decade of AIRE. Nat Rev Immunol. 2007;7:645–650.
[70] Mathis D, Benoist C. Aire. Annu Rev Immunol. 2009;27:287–312.
[71] Villaseñor J, Besse W, Benoist C, Mathis D. Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated. Proc Natl Acad Sci U S A. 2008;105:15854–15859.
[72] Rooke R, Waltzinger C, Benoist C, Mathis D. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. Immunity. 1997;7:123–134.
[73] Scollay R, Godfrey DI. Thymic emigration: conveyor belts or lucky dips? Immunol Today. 1995;16:268–274.
[74] Klein L. Dead man walking: how thymocytes scan the medulla. Nat Immunol. 2009;10:809–811.
[75] Koble C, Kyewski B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. J Exp Med. 2009;206:1505–1513.
[76] Millet V, Naquet P, Guinamard RR. Intercellular MHC transfer between thymic epithelial and dendritic cells. Eur J Immunol. 2008;38:1257–1263.
[77] Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol. 2005;6:331–337.
[78] Liu H, Leung BP. $CD4^+$ $CD25^+$ regulatory T cells in health and disease. Clin Exp Pharmacol Physiol. 2006;33:519–524.
[79] Cabarrocas J, Cassan C, Magnusson F, et al. $Foxp3^+$ $CD25^+$ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. Proc Natl Acad Sci U S A. 2006;30:8453–8458.
[80] Jordan MS, Boesteanu A, Reed AJ, et al. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. Nat Immunol. 2001;2:301–306.
[81] Caton AJ, Cozzo C, Larkin J, Lerman MA, Boesteanu A, Jordan MS. CD4(+) CD25(+) regulatory T cell selection. Ann N Y Acad Sci. 2004;1029:101–114.
[82] D'Cruz LM, Klein L. Development and function of agonist-induced $CD25^+Foxp3^+$ regulatory T cells in the absence of interleukin 2 signaling. Nat Immunol. 2005;6:1152–1159.
[83] Kim JM, Rudensky A. The role of the transcription factor Foxp3 in the development of regulatory T cells. Immunol Rev. 2006;212:86–98.
[84] Van Santen HM, Benoist C, Mathis D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. J Exp Med. 2004;200:1221–1230.
[85] Foy TM, Page DM, Waldschmidt TJ, et al. An essential role for gp39, the ligand for CD40, in thymic selection. J Exp Med. 1995;182:1377–1388.
[86] Page DM, Roberts EM, Peschon JJ, Hedrick SM. TNF receptor-deficient mice reveal striking differences between several models of thymocyte negative selection. J Immunol. 1998;160:120–133.
[87] Matzinger P, Guerder S. Does T-cell tolerance require a dedicated antigen-presenting cell? Nature. 1989;338:74–76.
[88] Kishimoto H, Sprent J. Several different cell surface molecules control negative selection of medullary thymocytes. J Exp Med. 1999;190:65–73.
[89] Kishimoto H, Sprent J. Negative selection in the thymus includes semimature T cells. J Exp Med. 1997;185:263–271.
[90] Kishimoto H, Surth CD, Sprent J. A role for Fas in negative selection of thymocytes in vivo. J Exp Med. 1998;187:1427–1438.

[91] Cloosen S, Arnold J, Thio M, Bos GM, Kyewski B, Germeraad WT. Expression of tumor-associated differentiation antigens, MUC1 glycoforms and CEA, in human thymic epithelial cells: implications for self-tolerance and tumor therapy. Cancer Res. 2007;67:3919–3926.
[92] Chentoufi AA, Palumbo M, Polychronakos C. Proinsulin expression by Hassall's corpuscles in the mouse thymus. Diabetes. 2004;53:354–359.
[93] Chentoufi AA, Polychronakos C. Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes. Diabetes. 2002;51:1383–1390.
[94] Brimnes MK, Jensen T, Jørgensen TN, Michelsen BK, Troelsen J, Werdelin O. Low expression of insulin in the thymus of non-obese diabetic mice. J Autoimmun. 2002;19:203–213.
[95] Giraud M, Taubert R, Vandiedonck C, et al. An IRF8-binding promoter variant and AIRE control CHRNA1 promiscuous expression in thymus. Nature. 2007;448:934–937.
[96] Anderson AC, Nicholson LB, Legge KL, Turchin V, Zaghouani H, Kuchroo VK. High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. J Exp Med. 2000;191:761–770.
[97] Klein L, Klugmann M, Nave KA, Tuohy VK, Kyewski B. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. Nat Med. 2000;6:56–61.
[98] Sakaguchi N, Takahashi T, Hata H, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature. 2003;426:454–460.
[99] Calanan B, Szychowski S, Chan FK, Cado D, Winoto A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. Immunity. 1995;3:273–282.
[100] Cavadini P, Vermi W, Facchetti F, et al. AIRE deficiency in thymus of 2 patients with Omenn syndrome. J Clin Invest. 2005;115:728–732.
[101] Honig M, Schwarz K. Omenn syndrome: a lack of tolerance on the background of deficient lymphocyte development and maturation. Curr Opin Rheumatol. 2006;18:383–388.
[102] Davies JK, Telfer P, Cavenagh JD, Foot N, Neat M. Autoimmune cytopenias in the 22q11.2 deletion syndrome. Clin Lab Haematol. 2003;25:195–197.
[103] Sakamoto O, Imaizumi M, Suzuki A, et al. Refractory autoimmune hemolytic anemia in a patient with chromosome 22q11.2 deletion syndrome. Pediatr Int. 2004;46:612–614.
[104] Sullivan KE, McDonald-McGinn DM, Driscoll DA, et al. Juvenile rheumatoid arthritis-like polyarthritis in chromosome 22q11.2 deletion syndrome (DiGeorge anomalad/velocardiofacial syndrome/conotruncal anomaly face syndrome). Arthritis Rheum. 1997;40:430–436.
[105] Brown JJ, Datta V, Browning MJ, Swift PG. Graves' disease in DiGeorge syndrome: patient report with a review of endocrine autoimmunity associated with 22q11.2 deletion. J Pediatr Endocrinol Metab. 2004;17:1575–1579.
[106] Jawad AF, McDonald-McGinn DM, Zackai E, Sullivan KE. Immunologic features of chromosome 22q11.2 deletion syndrome (DiGeorge syndrome/velocardiofacial syndrome). J Pediatr. 2001;139:715–723.
[107] Gennery AR. Antibody deficiency and autoimmunity in 22q11.2 deletion syndrome. Arch Dis Child. 2002;86:422–425.
[108] McLean-Tooke A, Spickett GP, Gennery AR. Immunodeficiency and autoimmunity in 22q11.2 deletion syndrome. Scand J Immunol. 2007;66:1–7.
[109] Kramradt T, Goggel R, Erb KJ. Induction, exacerbation and inhibition of allergic and autoimmune diseases by infection. Trends Immunol. 2005;26:260–267.
[110] McLean-Tooke A, Barge D, Spickett GP, Gennery AR. Immunologic defects in 22q11.2 deletion syndrome. J Allergy Clin Immunol. 2008;122:362–367.
[111] Nagamine K, Peterson P, Scott HS, et al. Positional cloning of the APECED gene. Nat Genet. 1997;17:393–398.
[112] Finnish-German APECED Consortium. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet. 1997;17:399–403.
[113] Aaltonen J, Horelli-Kuitunen N, Fan JB, et al. High-resolution physical and transcriptional mapping of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy locus on chromosome 21q22.3 by FISH. Genome Res. 1997;7:820–829.
[114] Ferguson BJ, Alexander C, Rossi SW, et al. AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. J Biol Chem. 2008;283:1723–1731.
[115] Pitkenen J, Rebane A, Rowell J, et al. Cooperative activation of transcription by autoimmune regulator AIRE and CBP. Biochem Biophys Res Commun. 2005;333:944–953.
[116] Ramsey C, Burkrinsky A, Peltonen L. Systematic mutagenesis of the functional domains of AIRE reveals their role in intracellular targeting. Hum Mol Genet. 2002;11:3299–3308.
[117] Gibson TJ, Ramu C, Gemund C, Aasland R. The APECED polyglandular autoimmune syndrome protein, AIRE-1, contains the SAND domain and is probably a transcription factor. Trends Biochem Sci. 1998;23:242–244.

[118] Ilmarinen T, Eskelin P, Halonen M, et al. Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation. Hum Mutat. 2005;26:322–331.
[119] Bottomlei MJ, Stier G, Pennacchini D, et al. NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1): insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. J Biol Chem. 2005;280:11505–11512.
[120] Bjorses P, Halonen M, Palvimo JJ, et al. Mutation in the AIRE gene: effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy protein. Am J Hum Genet. 2000;66:378–392.
[121] Meloni A, Incani F, Corda D, Cao A, Rosatelli MC. Role of PHD fingers and COOH-terminal 30 amino acids in AIRE transactivation activity. Mol Immunol. 2008;45:805–809.
[122] Halonen M, Kangas H, Ruppell T, et al. APECED-causing mutations in AIRE reveal the functional domains of the protein. Hum Mutat. 2004;23:245–257.
[123] Uchida D, Hatakeyama S, Matsushima A, et al. AIRE functions as an E3 ubiquitin ligase. J Exp Med. 2004;199:167–172.
[124] Savkur RS, Burris TP. The coactivator LXXLL nuclear receptor recognition motif. J Pept Res. 2004;63:207–212.
[125] Johnnidis JB, Venanzi ES, Taxman DJ, Ting JP, Benoist CO, Mathis DJ. Chromosomal clustering of genes controlled by the aire transcription factor. Proc Natl Acad Sci USA. 2005;17:7233–7238.
[126] Abramson J, Giraud M, Benoist C, Mathis D. Aire's partners in the molecular control of immunological tolerance. Cell. 2010;140:123–135.
[127] Pitkanen J, Vahamurto P, Krohn K, Peterson P. Subcellular localization of the autoimmune regulator protein: characterization of nuclear targeting and transcriptional activation domain. J Biol Chem. 2001;276:19597–19602.
[128] Kalkhoven E. CBP and p300: HATs for different occasions. Biochem Pharmacol. 2004;68:1145–1155.
[129] Peterson P, Org T, Rebane A. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. Nat Rev Immunol. 2008;8:948–957.
[130] Liiv I, Rebane A, Org T, et al. DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim Biophys Acta. 2008;1783:74–83.
[131] Ilmarinen T, Kangas H, Kytomaa T, et al. Functional interaction of AIRE with PIAS1 in transcriptional regulation. Mol Immunol. 2008;45:1847–1862.
[132] Oven I, Brdickova N, Khoutek J, Vaupotic T, Narat M, Peterlin BM. AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. Mol Cell Biol. 2007;27:8815–8823.
[133] Mellor J. It takes a PHD to read the histone code. Cell. 2006;126:22–24.
[134] Org T, Chignola F, Hetenyi C, et al. The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression. EMBO Rep. 2008;9:370–376.
[135] Koh AS, Kuo AJ, Park SY, et al. Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. Proc Natal Acad Sci USA. 2008;105:15878–15883.
[136] Cohen JN, Guidi CJ, Tewalt EF, et al. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. J Exp Med. 2010;207:681–688.
[137] Seach N, Ueno T, Fletcher AL, et al. The lymphotoxin pathway regulates Aire-independent expression of ectopic genes and chemokines in thymic stromal cells. J Immunol. 2008;180:5384–5392.
[138] Kim SH, Jeong JW, Park JA, et al. Regulation of the HIF-1alpha stability by histone deacetylases. Oncol Rep. 2007;17:647–651.
[139] Eldershaw SA, Sansom DM, Narendran P. Expression and function of the autoimmune regulator (Aire) gene in non-thymic tissue. Clin Exp Immunol. 2011;163:296–308.
[140] Wang CY, Shi JD, Davoodi-Semiromi A, She JX. Cloning of Aire, the mouse homologue of the autoimmune regulator (AIRE) gene responsible for autoimmune polyglandular syndrome type 1 (ASP1). Genomics. 1999;55:322–326.
[141] Mittaz L, Rossier C, Heino M, et al. Isolation and characterization of the mouse Aire gene. Biochem Biophys Res Commun. 1999;255:483–490.
[142] Kuroda N, Mitani T, Takeda N, et al. Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. J Immunol. 2005;174:1862–1870.
[143] Pöntynen N, Miettinen A, Arstila TP, et al. Aire deficient mice do not develop the same profile of tissue-specific autoantibodies as APECED patients. J Autoimmun. 2006;27:96–104.
[144] Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. J Exp Med. 1997;186:1677–1687.
[145] Smeltz RB, Wolf NA, Swanborg RH. Inhibition of autoimmune T cell responses in the DA rat by bone marrow-derived NK cells in vitro: implications for autoimmunity. J Immunol. 1999;163:1390–1397.

[146] Pignata C, Prasad KVS, Hallek M, et al. Phosphorylation of src family lck tyrosine kinase following interleukin-12 activation of human natural killer cells. Cell Immunol. 1995;165:211–216.
[147] Fort MM, Leach MW, Rennick DM. A role for NK cells as regulators of $CD4^+$ T cells in a transfer model of colitis. J Immunol. 1998;161:3256–3261.
[148] Aloj G, Giardino G, Valentino L, et al. Severe combined immunodeficiences: new and old scenarios. Int Rev Immunol. 2012;31:43–65.
[149] Mathew P, Crist WM, Furman WL. The use in cytokines in children. Curr Opin Pediatr. 1994;6:58–67.
[150] Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab. 2006;91:2843–2850.
[151] Betterle C, Greggio NA, Volpato M. Autoimmune polyglandular syndrome type 1. J Clin Endocr Metab. 1998;83:1049–1055.
[152] Ahonen P, Myllarniemi S, Sipila I, Perheentupa J. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med. 1990;322:1829–1836.
[153] Tóth B, Wolff AS, Halász Z, et al. Novel sequence variation of AIRE and detection of interferon-omega antibodies in early infancy. Clin Endocrinol (Oxf). 2010;72:641–647.
[154] Wolff AS, Erichsen MM, Meager A, et al. Autoimmune polyendocrine syndrome type 1 in Norway: phenotypic variation, autoantibodies, and novel mutations in the autoimmune regulator gene. J Clin Endocrinol Metab. 2007;92:595–603.
[155] Betterle C, Dal Pra C, Mantero F, Zanchetta R. Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev. 2002;23:327–364.
[156] Kisand K, Bøe Wolff AS, Podkrajsek KT, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. J Exp Med. 2010;207:299–308.
[157] Puel A, Döffinger R, Natividad A, et al. Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. J Exp Med. 2010;207:291–297.
[158] Husebye ES, Perheentupa J, Rautemaa R, Kämpe O. Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med. 2009;265:514–529.
[159] Bensing S, Brandt L, Tabaroj F, et al. Increased death risk and altered cancer incidence pattern in patients with isolated or combined autoimmune primary adrenocortical insufficiency. Clin Endocrinol (Oxf). 2008;69:697–704.
[160] Rautemaa R, Hietanen J, Niissalo S, Pirinen S, Perheentupa J. Oral and oesophageal squamous cell carcinoma—a complication or component of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, APS-I). Oral Oncol. 2007;43:607–613.
[161] Lankisch TO, Jaeckel E, Strassburg CP. The autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy or autoimmune polyglandular syndrome type 1. Semin Liver Dis. 2009; 29:307–314.
[162] Capalbo D, Mazza C, Giordano R, et al. Molecular background and genotype-phenotype correlation in APECED patients from Campania and in their relatives. J Endocrinol Invest. 2011; 35:169–173.
[163] Capalbo D, Elefante A, Spagnuolo MI, et al. Posterior reversible encephalopathy syndrome in a child during an accelerated phase of a severe APECED phenotype due to an uncommon mutation of AIRE. Clin Endocrinol (Oxf). 2008;69:511–513.
[164] Gavanescu I, Benoist C, Mathis D. B cells are required for Aire-deficient mice to develop multi-organ autoinflammation: a therapeutic approach for APECED patients. Proc Natl Acad Sci USA. 2008;105:13009–13014.
[165] Devoss JJ, Shum AK, Johannes KP, et al. Effector mechanisms of the autoimmune syndrome in the murine model of autoimmune polyglandular syndrome type 1. J Immunol. 2008;181:4072–4079.
[166] Zlotogora J, Shapiro MS. Polyglandular autoimmune syndrome type I among Iranian Jews. J Med Genet. 1992;29:824–826.
[167] Aaltonen J, Bjorses P, Sandkuijl L, Perheentupa J, Peltonen L. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. Nat Genet. 1994;8:83–87.
[168] Rosatelli MC, Meloni A, Devoto M, et al. A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. Hum Genet. 1998;103:428–434.
[169] Cetani F, Barbesino G, Borsari S, et al. A novel mutation of the autoimmune regulator gene in an Italian kindred with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, acting in a dominant fashion and strongly cosegregating with hypothyroid autoimmune thyroiditis. J Clin Endocr Metab. 2001;86:4747–4752.

[170] Podkrajses KT, Bratanic N, Krzisnik C, Battelino T. Autoimmune regulator-1 messenger ribonucleic acid analysis in a novel intronic mutation and two additional novel AIRE gene mutations in a cohort of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. J Clin Endocr Metab. 2005;90:4930–4935.
[171] Stolarski B, Pronicka E, Korniszewski L, et al. Molecular background of polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome in a Polish population: novel AIRE mutations and an estimate of disease prevalence. Clin Genet. 2006;70:348–354.
[172] Scott HS, Heino M, Peterson P, et al. Common mutations in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients of different origins. Mol Endocrinol. 1998;12:1112–1119.
[173] Pearce SH, Cheetham T, Imrie H, et al. A common and recurrent 13-bp deletion in the autoimmune regulator gene in British kindreds with autoimmune polyendocrinopathy type 1. Am J Hum Genet. 1998;63:1675–1684.
[174] Dominguez M, Crushell E, Ilmarinen T, et al. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in the Irish population. J Pediatr Endocrinol Metab. 2006;19:1343–1352.
[175] Wang CY, Davoodi-Semiromi A, Huang W, Connor E, Shi JD, She JX. Characterization of mutations in patients with autoimmune polyglandular syndrome type 1 (APS1). Hum Genet. 1998;103:681–685.
[176] Heino M, Scott HS, Chen Q, et al. Mutation analyses of North American APS-1 patients. Hum Mutat. 1999;13:69–74.
[177] Clemente MG, Meloni A, Obermayer-Straub P, Frau F, Manns MP, De Virgilis S. Two cytochromes P450 are major hepatocellular autoantigens in autoimmune polyglandular syndrome type 1. Gastroenterology. 1998;114:324–328.
[178] Meloni A, Willcox N, Meager A, et al. Autoimmune polyendocrine syndrome type 1: an extensive longitudinal study in Sardinian patients. J Clin Endocr Metab. 2012;97:1114–1124.
[179] Meloni A, Perniola R, Faa V, Corvaglia E, Cao A, Rosatelli MC. Delineation of the molecular defects in the AIRE gene in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients from Southern Italy. J Clin Endocrinol Metab. 2002;87:841–846.
[180] Cervato S, Mariniello B, Lazzarotto F, et al. Evaluation of the autoimmune regulator (AIRE) gene mutations in a cohort of Italian patients with autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) and in their relatives. Clin Endocrinol (Oxf). 2009;70:421–428.
[181] Giordano C, Modica R, Allotta ML, et al. Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) in Sicily: confirmation that R203X is the peculiar AIRE gene mutation. J Endocrinol Invest. 2011;35:384–388.
[182] Valenzise M, Wasniewska M, Mirabelli S, De Luca F, Cervato S, Betterle C. Identification of two novel mutations in the first Sicilian APECED patient with no R203X mutation in AIRE gene and review of Italian APECED genotypes. Gene. 2012;499:343–346.
[183] Betterle C, Ghizzoni L, Cassio A, et al. Autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) in Calabria: clinical, immunological and genetic patterns. J Endocrinol Invest. [Nov 21, 2011.Epub ahead of print].
[184] Ishii T, Suzuki Y, Ando N, Matsuo N, Ogata T. Novel mutations of the autoimmune regulator gene in two siblings with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocr Metab. 2000;85:2922–2926.
[185] Halonen M, Eskelin P, Myhre AG, et al. AIRE mutations and human leukocyte antigen genotypes as determinants of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy phenotype. J Clin Endocrinol Metab. 2002;87:2568–2574.
[186] Kekalainen E, Tuovinen H, Joensuu J, et al. A defect of regulatory T cells in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Immunol. 2007;178:1208–1215.
[187] Russell JH, Rush B, Weaver C, Wang R. Mature T cells of autoimmune lpr/lpr mice have a defect in antigen-stimulated suicide. Proc Natl Acad Sci U S A. 1993;90:4409–4413.
[188] Pignata C, Fiore M, De Filippo S, Cavalcanti M, Gaetaniello L, Scotese I. Apoptosis as a mechanism of peripheral blood mononuclear cell death following measles and varicella-zoster virus infections in children. Pediatr Res. 1998;43:77–83.
[189] Giardino G, Aloj G, Cirillo E, et al. Intergenerational anticipation of disease onset in people with multiple autoimmune syndrome. Diabetes Res Clin Pract. 2011;94:37–39.

Review

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EXPERT REVIEWS

Alterations of the autoimmune regulator transcription factor and failure of central tolerance: APECED as a model

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Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Central tolerance within the thymus and peripheral tolerance in peripheral lymphoid organs lead to immunologic nonresponsiveness against self-components. The central tolerance represents the mechanism by which T cells binding with high avidity to self-antigens are eliminated through the so-called negative selection. Thymic medullary epithelial cells and medullary dendritic cells play a key role in this process, through the expression of a large number of tissue-specific self-antigens involving the transcription factor autoimmune regulator (AIRE). Mutations of *AIRE* result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, a rare autosomal recessive disease (OMIM 240300), which is the paradigm of a genetically determined failure of central tolerance and autoimmunity. This review focuses on recent advances in the molecular mechanisms of central tolerance, their alterations and clinical implication.

KEYWORDS: AIRE • autoimmunity • autoimmune polyendocrinopathy candidiasis ectodermal dystrophy • central tolerance • phenotypic variability • susceptibility genes

The thymus represents the central organ of immunologic self–nonself discrimination. Within the thymus, developing thymocytes undergo positive and negative selection, resulting in mature T cells that are able to successfully recognize a wide range of foreign antigens and, at the same time, able to ignore self-antigens. Potentially, autoreactive naive T cells, which succeed in reaching the periphery, are functionally suppressed by several mechanisms, in a process known as peripheral tolerance. Breakdown of the central or peripheral tolerance leads to the development of destructive autoimmune reactions and, paradoxically, in a few circumstances also to immunodeficiency, as seen in experimental conditions regarding alteration of the transcription factor autoimmune regulator (AIRE) protein or FOXP3. Recent evidence suggests that systemic autoimmunity and immunodeficiency can be strictly interconnected. In immunodeficiencies, the decreased or abolished capacity of the immune system to clear infections causes a continuous immunological stimulation and activation, which represents one of the potential mechanisms that eventually leads to autoimmunity. Dysregulation of immunoregulatory factors combined to multiple genetic variations is responsible for systemic autoimmunity. In a few cases, along with a predominant autoimmune phenotype, an increased susceptibility to infections is also present [1].

Several studies have reported evidence indicating that autoimmune phenomena occur in patients affected with primary immunodeficiencies, and have focused on the molecular and cellular mechanisms underlying these conditions, in order to elucidate the link between autoimmunity and immunodeficiency. On the other hand, studies on genetic autoimmune disorders are also extremely useful for our understanding of the pathophysiology of the intimate mechanisms that lead autoreactive clones to escape clonal deletion [2,3].

Currently, unique models, which help unravel many aspects of the development, homeostasis and regulatory properties of the immune system, are the monogenic autoimmune disorders and, in particular, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), immunodysregulation, polyendocrinopathy and enteropathy X-linked (IPEX), autoimmune

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lymphoproliferative syndrome and IL-2 receptor α-chain (IL-2RA, CD25) deficiency.

Impairment of central or peripheral tolerance, which normally prevents the survival, expansion and activation of autoreactive T cells, thus protecting from autoimmune diseases, is the main pathogenetic mechanism responsible for these conditions. Since an important contribution to our understanding of autoimmunity was given by genetic models of abnormal central tolerance, in this review, the authors focus on APECED pathogenesis, a paradigmatic example of a disease due to the failure of central tolerance.

Functional & molecular mechanisms governing the central tolerance

Positive & negative selection

Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The immune system is able to discriminate between self- and nonself-antigens. This property is fundamental to induce a productive immunity and to prevent autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. Immune tolerance to self-antigens is acquired through two main processes: central and peripheral tolerance [4]. Central tolerance takes place within the thymus through the negative selection process, which occurs subsequently to the positive one. Eventually, the tolerance to self-antigens expressed in various tissues is acquired [4].

T-cell precursors originate in the bone marrow, like B lymphocytes, but, unlike these cells, complete their development within the thymus. After entering the thymus, immature lymphocytes make a commitment to the αβ T-cell lineage, rearranging first the T-cell receptor (TCR)β chain and then the TCRα chain in a sequential manner. At this stage, developing double-positive (DP) thymocytes, expressing both CD4 and CD8 on the cell surface, with functional TCR can be positively selected by peptide–MHC in the thymic cortex. The majority of DP thymocytes do not receive a rescue signal through TCR and, therefore, undergo 'death by neglect' or apoptosis. Only approximately 5% of DP cells are able to bind an MHC ligand with mild avidity, inducing their maturation to the $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) cells (Figure 1) [5]. Positively selected DP cells trigger the expression of chemokines that direct the T cells through the subsequent maturation step within the thymic medulla [6]. Here, the last stage of T-cell development takes place, the so-called negative selection, a fundamental process to establish central tolerance. This process enables T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, to be eliminated (Figure 1) [4,7]. Subsequently, thanks to the mechanisms of peripheral tolerance, autoreactive lymphocytes that have escaped negative selection are deleted in the periphery [4].

Within the thymic medulla, thymic medullary epithelial cells (mTECs) and medullary dendritic cells (mDCs) play a central role in the establishment of self-tolerance through the negative selection process [8]. Both cellular types, expressing the costimulatory molecules, CD40, CD80, CD86 and MHC class II, are able to favor an efficient thymocyte deletion. mTECs express a thousand genes, the so-called 'promiscuous gene expression', including tissue-specific self-antigens (TSAs), normally present only in specialized peripheral organs [9]. This high expression by mTECs of genes encoding TSAs is driven by AIRE [10–12]. TSAs include, among other tissues, proteins restricted to the pancreas, stomach, eye, salivary gland, muscle and thyroid [10,13,14]. These MHC-restricted self-antigens are presented by mTECs to developing T cells. Thymocytes that recognize self-antigens with high affinity/avidity are deleted [15]. Remarkably, not all TSAs seem to be regulated by AIRE, since the expression of some of them, such as acid decarboxylase (GAD67), is not altered in AIRE deficiency. In fact, AIRE controls TSAs' gene expression to variable degrees, some genes being strongly dependent on its activity, while others weakly or not at all dependent, such as C-reactive protein and GAD67. Although no definitive explanation for this phenomenon is available, certainly, further genetic and/or epigenetic mechanisms might be involved in the regulation of the complex promiscuous gene-expression process in mTECs. Unfortunately, to date, they still remain unraveled, in spite of the increasing knowledge of the AIRE mechanism of action. It is surprising that a low number of mTECs are able to delete such a huge number of clonotypes, since only a few mTECs express a given TSA [11,12]. A possible explanation is that during a 4- to 5-day period, there is a high motility of thymocytes, allowing a huge number of interactions with mTECs [16]. mDCs have a similar role, although the expression of self-antigens on the cell surface is mostly dependent on the phagocytosis of apoptotic mTECs [17,18].

Some of the thymocytes, which bind self-MHC–peptide complexes with high affinity, express *Foxp3*, and through a yet unknown mechanism escape negative selection and differentiate into Tregs, which play an important role in the peripheral tolerance suppressing autoreactive T cells in the periphery [19,20].

Triggering the costimulatory molecules and the subsequent activation of Fas signaling pathway represents the commonly accepted mechanism to explain the deletion of self-reactive thymocytes within the thymus [21]. Indeed, the costimulatory molecules expressed by mTECs and mDCs, such as CD40, CD80 and CD86, are involved in the process [22]. Moreover, B7 molecules, which interact with thymocyte CD28 receptor and are expressed by antigen presenting cells, also represent a crucial costimulatory factor [23]. Indeed, studies have shown that exposure of DP thymocytes to anti-TCR monoclonal antibody (mAb) fails to induce thymocyte death, but not in the presence of antigen presenting cells or in the combined presence of anti-TCR and anti-CD28 mAb *in vitro* [23]. Furthermore, it has been observed in SP thymocyte cultures that the exposure to high concentrations of anti-TCR mAbs is able to induce apoptosis through high-level TCR signaling in the absence of any costimulation [23]. Thus, in this condition, the strong TCR ligation leads to apoptosis in a Fas-dependent manner [24]. This effect is abolished in Fas-lpr/lpr cells [24], carrying homozygous mutation of Fas. Moderate-avidity TCR binding with costimulatory molecules induces cell death in a Fas-independent manner.

AIRE

In humans, the *AIRE* gene maps to chromosome 21q22.3 and has been cloned simultaneously in 1997 by two independent research groups [25,26]. It consists of 14 exons spanning 11.9 kb of genomic DNA [27] and encodes a 545 amino-acid protein with a molecular weight of approximately 58 kDa [25].

The AIRE protein is mainly localized in the cell nucleus, within nuclear bodies distributed near to nuclear speckles [28], suggesting the involvement of AIRE in the regulation of gene transcription. This hypothesis has also been confirmed by structural characteristics of the protein. Three different isoforms of AIRE protein, generated by alternative splicing, have been identified. These different isoforms show a unique combination of domains, common to transcriptional regulators and chromatin-binding proteins. The N-terminal region shows a six-helix structure with high similarity to a caspase-recruitment domain [29], which is necessary for AIRE oligomerization and localization to promyelocytic leukemia nuclear domains [30]. Moreover, AIRE contains a Sp100, AIRE1, NucP41/75, DEAF1 (SAND) domain [31] important for AIRE transactivation capacity and subcellular localization [32]. The C-terminal region contains two plant homeodomain PHD-type zinc fingers [33], which, if mutated induce a severe decrease of AIRE transcriptional activation capacity [34]. Other structural features include a conserved nuclear targeting signal, four LXXLL motifs or nuclear receptor interaction domains, which are found on coactivators of nuclear receptors and proline-rich regions and are also associated to transcription regulation [35].

In order to study the function of AIRE, transgenic mouse models were generated. The murine *Aire* gene has been mapped to chromosome 10. It shows a strict similarity of the genetic sequence and structural organization with the human ortholog [36]. Studies of Aire knockout mice ($Aire^{-/-}$) have proved to be an important tool in furthering our understanding of how *Aire* prevents autoimmunity. The gene-expression profile of mTECs from $Aire^{-/-}$ has shown a reduced expression of many TSAs, compared with wild-type mice. Accordingly, $Aire^{-/-}$ mice show several autoimmune manifestations, lymphocytic infiltrates in multiple organs and autoantibodies against several peripheral organs and tissues, including the salivary gland, retina, pancreas, stomach, ovary and thyroid [37]. However, $Aire^{-/-}$ mice do not exhibit increased susceptibility to *Candida* infections [38].

Nevertheless, the precise molecular mechanisms that AIRE uses to regulate transcription remain to be clarified. AIRE acts as a coactivator in a large transcriptional complex, since the vast majority of *AIRE*-regulated genes are arranged in chromosomal clusters [39]. Moreover, recent studies have demonstrated that AIRE binds a large array of partners (Figure 2) [40]. AIRE's partners could be divided into four major functional classes: nuclear transport, chromatin binding/structure, transcription and pre-mRNA processing factors [40]. The first protein reported to bind to AIRE was CREB-binding protein [41]. CREB-binding protein acetylates both histone and nonhistone proteins and is important for the initiation of transcription. After its interactions with AIRE, histone acetylation and recruitment of chromatin transcription factors may promote gene-transcription activation [42,43]. AIRE's other partners have been identified, such as DNA protein kinase (DNA-PK) and SP-RING domain protein inhibitor of activated STAT1 (PIAS1) [44,45]. DNA-PK is a serine/threonine kinase activated by dsDNA breaks, which plays a key role in VDJ recombination [46]. DNA-PK deficiency in the stromal compartment leads to decreased TSA expression in mTECs, thus resulting in autoantibody production [44]. AIRE also binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II. It represents a key element enhancing the transcription–elongation process [47]. It is plausible that AIRE recruitment of all its cotranscriptional partners to TSA gene promoters, which are otherwise silenced, activates the expression of the large number of TSA in mTECs [48].

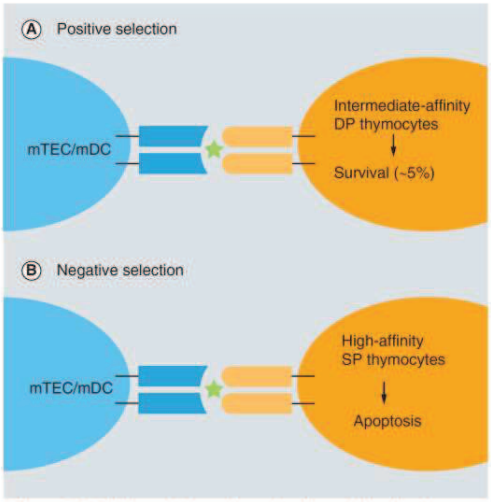

Figure 1. Positive and negative selection of developing thymocytes. (A) Positive selection of DP thymocytes, which bind with intermediate-affinity self-antigens presented by mTECs and mDCs. **(B)** Negative selection of SP thymocytes binding with high-affinity self-antigens.
DP: Double positive; mDC: Medullary dendritic cell; mTEC: Thymic medullary epithelial cell; SP: Single positive.

Accumulating evidence suggests that Aire may also play a role in the proper differentiation of the thymic medullary epithelium. Aire has been shown to be able to induce apoptosis in end-stage terminally differentiated mTECs ($K5^-K8^+$) [49]. Stellate $K5^+$ mTECs are normally eliminated by Aire's proapoptotic activity before completion of their terminal differentiation, and only an absence of Aire would reveal the full program of mTEC terminal differentiation ending with the globular $K5^-K8^+$. Aire may also promote mTECs' differentiation program. Evidences suggest that lack of Aire in mTECs results in an arrest of the differentiation program, with the cells remaining at the premature stage just before terminal differentiation. The $Aire^{-/-}$ mTECs lack the transcriptional activity for Aire-dependent TSA genes [50].

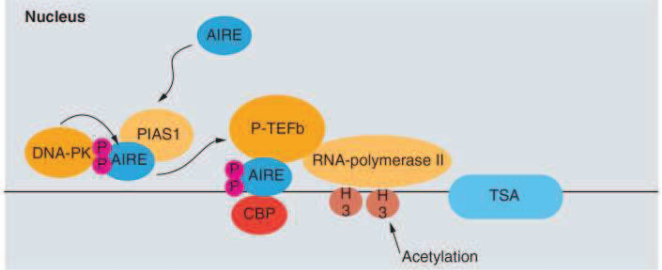

Figure 2. Autoimmune regulator's partners in the transcription regulation of tissue-specific self-antigens.
TSA: Tissue-specific self-antigen.

The *AIRE* gene is also expressed in peripheral lymphoid tissues, such as lymph nodes and spleen, at a lower degree than in thymic stromal cells. Recent studies have identified a population of extrathymic *Aire*-expressing cells in lymph nodes, corresponding to a subset of activated dendritic cells, which express TSAs and tolerogenic molecules. Presumably, also in the periphery, AIRE contributes to the process of immune tolerance by inducing TSAs' gene expression [15,51].

Alterations of AIRE expression have also been detected in patients with immunodeficiency and autoimmunity [46]. A severe reduction of AIRE mRNA and protein has been found in the thymus of two Omenn syndrome patients and one patient with T–B–NK$^+$ SCID. In addition, there was no detectable mRNA for the self-antigens insulin, CYP450 1A2, or fatty acid-binding protein in the immunodeficient patients. These findings led the authors to argue that deficiency of AIRE expression occurs in severe immunodeficiencies, characterized by abnormal T-cell development, and suggested that in Omenn syndrome, the few residual T-cell clones may have escaped negative selection and, thereafter, may eventually have expanded in the periphery, thus causing massive autoimmune reactions [52].

Central tolerance failure: the APECED model

APECED represents the prototypic disease of immune dysregulation, classified as a type IV immunodeficiency [53]. It is a rare autosomal recessive disease (OMIM 240300) caused by mutations in the *AIRE* gene [25,26] with a complex clinical phenotype discovered over decades.

Although rare, APECED has been reported worldwide [54] with a wide variability in its incidence. Epidemiologic data show a high prevalence in certain genetically isolated populations, such as Iranian Jews (1:9000) [55], Finns (1:25,000) [56] and Sardinians (1:14,400) [57]. It is also quite frequent in Norway [58] and Italy [59]. So far, more than 60 different types of mutations in the *AIRE* gene have been reported in APECED patients [14]. Different mutations have been found to be peculiar to specific areas. In Italy, an increased APECED prevalence has been found in various regions, in particular in Sardinia, Apulia and in the Venetian area. Furthermore, in both Sardinia and Apulia, two peculiar mutations of *AIRE* have been identified: the mutation R139X on exon 3 in Sardinia [60] and the W78R mutation on exon 2 in Apulia [61]. In the Veneto region, differing from the other Italian regions, *AIRE* mutations (R257X on exon 6 and 8) were similar to that identified in Finnish and Anglo-Saxon patients [62]. Recently, a typical mutation has also been identified in Sicily (R203X on exon 5) [63]. The patients from Campania show a high frequency of mutations in the exon/intron 1 junction [59].

APECED is characterized by destructive autoimmune reactions against endocrine and nonendocrine tissues and, frequently, ectodermal tissues. However, a high variable clinical expression has been well documented. Parathyroid glands, adrenal cortex, gonads, pancreatic β cells, gastric parietal cells and thyroid glands are mainly involved in the pathologic process. A few ectodermal manifestations in APECED are dental enamel hypoplasia, pitted nail dystrophy and alopecia. Furthermore, the gastrointestinal system may also be involved, leading to autoimmune gastritis, malabsorption and autoimmune hepatitis [54]. The clinical diagnosis requires the presence of at least two of the three most common clinical features, the classical triad: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH) and Addison's disease (AD) [64]. However, if a sibling has already been diagnosed, the presence of even one component is sufficient for the diagnosis [65]. Molecular analysis of the *AIRE* gene is necessary to confirm the diagnosis and, in addition, can be helpful in those cases with atypical clinical presentation. Another specific and sensitive diagnostic tool for APECED, recently discovered, is represented by autoantibodies (autoAbs) neutralizing cytokines, in particular, type I interferons (IFN-ω and IFN-α) and Th17-related cytokines (IL-17A, IL-17F and IL-22), which emerge in an early phase of the disease, even before any clinical manifestation [66]. Organ-specific autoAbs are not used in the diagnostic procedure because of their later appearance, generally at the meantime of the corresponding autoimmune manifestation. Moreover, they are detected in 8–66% of APECED patients and are mostly not APECED-specific, but specific to the individual disease [67]. In most patients, CMC precedes the other immune disorders appearing by the age of 5 years, usually followed by CH and later by AD [68]. While a clear autoimmune pathogenesis underlies the endocrine disorders pathology, which results from destruction of the target organ by the cellular and antibody-mediated attack [65], the molecular basis of CMC and of the increased susceptibility to infections in APECED patients is still poorly understood. Indeed, autoAbs to parathyroid, adrenal glands and type I interferons are hallmarks of APECED [34,69]. Therefore, the high prevalence and early appearance of anticytokine autoAbs is very suggestive of a role in the pathogenesis of immunodeficiency. Recent studies have described a role of the autoAbs against IL-22, IL-17A and IL-17F cytokines in the pathogenesis of CMC [70,71]. CMC is a clinical feature shared by distinct

genetic diseases affecting the differentiation or functionality of Th17 lymphocytes, such as hyper-IgE syndrome. Remarkably, autoAbs against Th17-related cytokines have also been found in rare thymoma patients presenting with CMC. Of note, in the vast majority of thymomas, the neoplastic mTECs fail to express AIRE [72]. In APECED patients, IL-17F and IL-22 secretion, in particular, seems to be significantly reduced in response to *Candida albicans* [70]. All these findings allow us to speculate that CMC in APECED is also essentially autoimmune in nature and related to the development of an autoimmune response towards the IL-17 and IL-22 producing cells [73].

The current model of disease pathogenesis of APECED is based on the transgenic mouse models that showed impaired negative selection of autoreactive thymocytes in an Aire-deficient thymus. Aire deficiency in mTECs impaired the expression of a wide array of self-tissue specific antigens, which are not presented to differentiating T lymphocytes. As a consequence, potentially autoreactive T cells do not undergo negative selection and are exported to the periphery. Here, the naive autoreactive lymphocytes must also escape peripheral tolerance mechanisms to become dangerous.

Recent studies have highlighted some discrepancies in this pathogenetic model. In fact, it cannot explain the reason by which the organs affected are limited mainly to endocrine and ectodermal tissues, while AIRE controls the expression of thousands of peripheral antigens in the thymus. In addition, it should be noted that the first autoAbs to emerge in APECED patients are those against cytokines and not autoAbs implicated in organ-specific autoimmunity. Notably, cytokine antigens are not expressed by mTECs and are, therefore, not AIRE dependent [66]. According to the current model, breaking of peripheral tolerance mechanisms is also required to activate naive and autoreactive T cells reaching the periphery. All these steps need a period of time, which contrasts with the early onset of CMC, CH and AD, also taking into account the delay between initial autoimmunization and clinical manifestation, which appear only after almost a total destruction of the parathyroids or adrenal cortex occurs [74]. To explain these discrepancies, the authors have hypothesized a novel pathogentic model of 'active intrathymic autoimmunization', which alludes at additional roles for AIRE beyond regulating TSA expression [73]. According to this model, AIRE deficiency impairs the thymic microenvironment, leading to the formation of tertiary lymphoid tissue. Thymus thus becomes a site of immunization where cytokines are presented in an immunologic manner to T and B cells. Neutralizing autoantibodies against cytokines and activated autoreactive T-cells are then released directly from the thymus. After thymic export, they are already able to attack their targets [73]. Moreover, activated autoreactive T-cells against parathyroids and adrenal glands could be generated early because of the presence of parathyroid and adrenal antigens in the normal thymus. Indeed, parathyroids and thymus share the same embryological origins and it is not rare to find ectopic parathyroid tissue within the thymus. Concerning adrenal auto-antigens, several studies have reported the presence of paracrine glucocorticoid activity in the thymus. These auto-antigens should be presented by mDCs in the tertiary lymphoid infiltrates. To date, cell types and mechanisms responsible for this process remain unraveled. The two pathogenetic models are not mutually exclusive, but can coexist, the novel model explaining the earlier and most prevalent manifestations, such as autoAb responses, CMC, CH and AD (and probably gonadal failure too), and the current one explaining the later clinical features, such as diabetes, thyroid disease among others. Nevertheless, further studies are necessary to validate both the current and the emerging models of disease pathogenesis in APECED.

Phenotypic variability & genetic susceptibility

Patients affected with APECED usually show a variable clinical phenotype, in spite of the fact that APECED is a monogenic disorder. Many authors have confirmed this phenotypic and course heterogeneity among several populations, first documented in the largest reported series of 91 Finnish patients [54,56,57,59]. A precise genotype–phenotype correlation is still lacking. Notably, the clinical variability is both interfamilial and intrafamilial, the clinical features varying not only among patients from different families but also among siblings carrying the same mutation [59]. Different genetic and nongenetic factors, including modifier genes, chance and environmental interactions, as well as immune system dynamics, have a key role in modulating the clinical expression of the syndrome, even in the same family, thus explaining the intrafamilial and interfamilial variability.

Additional genetic loci, in particular the *HLA*s, have been shown to be related to certain phenotypic manifestations of APECED [14,75]. In particular, specific *HLA* haplotypes have been found to be associated to alopecia, AD and autoimmune thyroiditis in APECED patients. However, the *HLA* alleles do not have a strong influence on autoantibody formation, since only a weak association has been observed between the HLA type and the autoantibody specificities in APECED patients [75]. Several peripheral mechanisms of tolerance are also involved in the control and regulation of immune response, thus influencing the clinical expression of the disease. Natural killer cell activity represents an additional mechanism of peripheral tolerance involved in controlling the reactivity to self-antigens in the periphery. To date, because of the few studies, not much is known on the functionality of these immunological tolerance mechanisms in patients with APECED. Recently, a defect of $CD4^+CD25^+$ Tregs in APECED patients has also been reported [76]. It should be pointed out that a reduction of Tregs in APECED patients could also be observed as a secondary effect of chronic fungal infection in these individuals. On the contrary, in a recent study, an imbalance of the IL-7–IL-7R pathway leading to a loss of $CD8^+$ T-cells homeostasis has been documented in APECED patients. The abnormalities do not seem to be related to the inflammation itself. The decrease of CD127 and CD5 surface expression, along with increased levels of perforin in CD8RA cells, seems to be crucial in the breakdown of the tolerance in APECED patients [77].

Recently, two siblings affected with APECED, carrying the same mutation of *AIRE* (IVS1 + 1G>C; IVS1 + 5delG mutation), were investigated in order to detect genetic, environmental

and molecular factors potentially responsible of the intrafamilial phenotypic variability of APECED. Indeed, they were characterized by an extremely different phenotypic expression despite the identical mutation [59]. The younger sister, in particular, had a mild form of the syndrome, while the older male developed a severe phenotype exhibiting an accelerated phase involving parathyroid, thyroid, oral mucosa, skin, liver, adrenal glands, bowel and stomach, culminating in a life-threatening posterior encephalopathy syndrome never described before in the context of APECED [78]. APECED, different from the other autoimmune diseases, shows the same incidence between males and females and, so far, there is no evidence that the severity of the disease is influenced by sex. Exposure to infectious triggers (rubella, Epstein–Barr virus, cytomegalovirus, toxoplasma, varicella zoster virus, parvovirus B19, herpes simplex virus and parainfluenza virus) [79], autoantibody profile, mechanisms of peripheral tolerance (Fas-induced apoptosis, number of $TCD4^+CD25^+$ regulatory cells and natural killer activity) and HLA haplotype were compared in the two siblings. The clinical variability observed between the two siblings is not related to the differences in the exposure to infectious agents or functionality of mechanisms governing peripheral tolerance. This evidence suggests that the phenotypic variability of APECED may derive from the interaction between multiple genetic, epigenetic and environmental factors.

Expert commentary & five-year view

Recent evidence indicates that systemic autoimmunity and immunodeficiency can be strictly linked. Molecular mechanisms involved in central tolerance, along with those in peripheral tolerance, play a crucial role in the establishment and maintenance of immune self-tolerance, preventing autoimmunity and promoting the proper function of immune system. The study of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms give an enormous contribution to the elucidation of the molecular basis of human autoimmune disorders, generally considered as multifactorial diseases. APECED represents a paradigmatic example of monogenic disease due to mutations of the autoimmune regulator AIRE, which leads to central tolerance failure. This disease has been extremely useful for our understanding of the functional and molecular events that lead to the elimination of self-reactive T-cell clones within the thymus. Experimental evidence indicates that AIRE acts as a transcription factor, which upregulates the expression of TSA in mTECs allowing the negative selection of potentially autoreactive T-cells. The recent discovery of autoAbs directed towards Th17-related cytokines suggested an additional role for AIRE, which leads to argue a parallel pathogenetic hypothesis for APECED that also explains CMC pathogenesis, representing a puzzle to date. Moreover, evidence that IL-22 and IL-17F are crucial in protection against CMC infection has important therapeutic implications. The elucidation that TSAs are broadly expressed by mTECs also helped understand the mechanism by which autoimmunity may also be paradoxically associated with an immunodeficiency status. However, much more remains to be learned about the role of AIREs and its precise mechanism of action. Further studies are needed to improve our understanding of the molecular mechanisms of central tolerance involving the AIRE protein. This future research is not only important for the basic understanding, but may also have important clinical and therapeutic implications. Indeed, additional proteins or pathways may be identified, which could be used as diagnostic tools or as a target of novel therapeutic interventions to prevent autoimmunity.

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Key issues

- Immune self-tolerance represents a state of immunologic nonresponsiveness against the organism's own components. It is a fundamental property of the immune system, which prevents autoimmunity.
- Central tolerance mechanisms are the processes through which immune self-tolerance is established within the thymus.
- Negative selection is considered the main mechanism involved in central tolerance, by which potentially autoreactive T-cells bind self-antigens with high avidity and are eliminated.
- Autoimmune regulator protein acts as a transcription factor, which regulates the promiscuous thymic expression of tissue-specific self-antigens by thymic medullary epithelial cells and antigen presenting cells.
- Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is the result of mutations in the *AIRE* gene and represents the prototypic monogenic disease due to a failure of the central tolerance mechanisms.
- The pathogenetic mechanisms of APECED are, however, not completely understood. Recent evidence has shown a presumptive role of autoantibodies anti-Th17-related cytokines in APECED pathogenesis.
- The novel theory leads us to hypothesize a new pathogenesis based on an 'active intrathymic autoimmunization', which alludes at additional roles for autoimmune regulator protein.
- Further studies are required to define in detail APECED pathogenesis, which could have remarkable clinical relevance, both in diagnostics and therapeutics.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Grammatikos AP, Tsokos GC. Immunodeficiency and autoimmunity: lessons from systemic lupus erythematosus. *Trends Mol. Med.* 18(2), 101–108 (2012).

2 Pessach IM, Notarangelo LD. X-linked primary immunodeficiencies as a bridge to better understanding X-chromosome related autoimmunity. *J. Autoimmun.* 33(1), 17–24 (2009).

3 Pignata C, Alessio M, Ramenghi U *et al.* Clustering of distinct autoimmune diseases associated with functional abnormalities of T cell survival in children. *Clin. Exp. Immunol.* 121(1), 53–58 (2000).

4 Griesemer AD, Sorenson EC, Hardy MA. The role of the thymus in tolerance. *Transplantation* 90(5), 465–474 (2010).

• **Describes the role of the thymus in T-cell development and in the induction of tolerance.**

5 Cheroutre H. Starting at the beginning: new perspectives on the biology of mucosal T-cells. *Annu. Rev. Immunol.* 22, 217–246 (2004).

6 Brewer JA, Kanagawa O, Sleckman BP, Muglia LJ. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids *in vivo*. *J. Immunol.* 169(4), 1837–1843 (2002).

7 Palmer E. Negative selection – clearing out the bad apples from the T-cell repertoire. *Nat. Rev. Immunol.* 3(5), 383–391 (2003).

•• **Illustrates several cellular and molecular aspects of negative selection.**

8 Kyewski B, Klein L. A central role for central tolerance. *Annu. Rev. Immunol.* 24, 571–606 (2006).

9 Derbinski J, Gäbler J, Brors B *et al.* Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J. Exp. Med.* 202(1), 33–45 (2005).

10 Anderson MS, Venanzi ES, Klein L *et al.* Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298(5597), 1395–1401 (2002).

11 Derbinski J, Pinto S, Rösch S, Hexel K, Kyewski B. Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. *Proc. Natl Acad. Sci. USA* 105(2), 657–662 (2008).

12 Gillard GO, Farr AG. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J. Immunol.* 176(10), 5815–5824 (2006).

13 Kyewski B, Peterson P. Aire, master of many trades. *Cell* 140(1), 24–26 (2010).

•• **Discusses the AIRE's potential mechanisms of action on the basis of its interaction with other transcription factors.**

14 Mathis D, Benoist C. Aire. *Annu. Rev. Immunol.* 27, 287–312 (2009).

15 Gardner JM, Devoss JJ, Friedman RS *et al.* Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science* 321(5890), 843–847 (2008).

16 Rooke R, Waltzinger C, Benoist C, Mathis D. Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. *Immunity* 7(1), 123–134 (1997).

17 Klein L. Dead man walking: how thymocytes scan the medulla. *Nat. Immunol.* 10(8), 809–811 (2009).

• **Description of how developing T-cells scan the thymic microenvironment in order to acquire immune self-tolerance.**

18 Koble C, Kyewski B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J. Exp. Med.* 206(7), 1505–1513 (2009).

19 Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat. Immunol.* 6(4), 331–337 (2005).

20 Cabarrocas J, Cassan C, Magnusson F *et al.* Foxp3$^+$ CD25$^+$ regulatory T-cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl Acad. Sci. USA* 103(22), 8453–8458 (2006).

21 DeFranco S, Bonissoni S, Cerutti F *et al.* Defective function of Fas in patients with type 1 diabetes associated with other autoimmune diseases. *Diabetes* 50(3), 483–488 (2001).

22 Foy TM, Page DM, Waldschmidt TJ *et al.* An essential role for gp39, the ligand for CD40, in thymic selection. *J. Exp. Med.* 182(5), 1377–1388 (1995).

23 Kishimoto H, Sprent J. Several different cell surface molecules control negative selection of medullary thymocytes. *J. Exp. Med.* 190(1), 65–73 (1999).

24 Kishimoto H, Surh CD, Sprent J. A role for Fas in negative selection of thymocytes *in vivo*. *J. Exp. Med.* 187(9), 1427–1438 (1998).

25 Nagamine K, Peterson P, Scott HS *et al.* Positional cloning of the *APECED* gene. *Nat. Genet.* 17(4), 393–398 (1997).

26 Consortium F-GA. An autoimmune disease, *APECED*, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet.* 17(4), 399–403 (1997).

27 Aaltonen J, Horelli-Kuitunen N, Fan JB *et al.* High-resolution physical and transcriptional mapping of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy locus on chromosome 21q22.3 by FISH. *Genome Res.* 7(8), 820–829 (1997).

28 Björses P, Halonen M, Palvimo JJ *et al.* Mutations in the *AIRE* gene: effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy protein. *Am. J. Hum. Genet.* 66(2), 378–392 (2000).

29 Ferguson BJ, Alexander C, Rossi SW *et al.* AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. *J. Biol. Chem.* 283(3), 1723–1731 (2008).

30 Ramsey C, Bukrinsky A, Peltonen L. Systematic mutagenesis of the functional domains of AIRE reveals their role in intracellular targeting. *Hum. Mol. Genet.* 11(26), 3299–3308 (2002).

31 Gibson TJ, Ramu C, Gemünd C, Aasland R. The APECED polyglandular autoimmune syndrome protein, AIRE-1, contains the SAND domain and is probably a transcription factor. *Trends Biochem. Sci.* 23(7), 242–244 (1998).

32 Ilmarinen T, Eskelin P, Halonen M *et al.* Functional analysis of SAND mutations in *AIRE* supports dominant inheritance of the G228W mutation. *Hum. Mutat.* 26(4), 322–331 (2005).

33 Bottomley MJ, Stier G, Pennacchini D *et al.* NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. *J. Biol. Chem.* 280(12), 11505–11512 (2005).

34 Meloni A, Incani F, Corda D, Cao A, Rosatelli MC. Role of PHD fingers and COOH-terminal 30 amino acids in AIRE transactivation activity. *Mol. Immunol.* 45(3), 805–809 (2008).

35 Savkur RS, Burris TP. The coactivator LXXLL nuclear receptor recognition motif. *J. Pept. Res.* 63(3), 207–212 (2004).

▶36 Wang CY, Shi JD, Davoodi-Semiromi A, She JX. Cloning of Aire, the mouse homologue of the autoimmune regulator (*AIRE*) gene responsible for autoimmune polyglandular syndrome type 1 (ASP1). *Genomics* 55(3), 322–326 (1999).

▶37 Kuroda N, Mitani T, Takeda N *et al.* Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of *Aire*-deficient mice. *J. Immunol.* 174(4), 1862–1870 (2005).

▶38 Hubert FX, Kinkel SA, Crewther PE *et al.* *Aire*-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype. *J. Immunol.* 182(6), 3902–3918 (2009).

▶39 Johnnidis JB, Venanzi ES, Taxman DJ, Ting JP, Benoist CO, Mathis DJ. Chromosomal clustering of genes controlled by the aire transcription factor. *Proc. Natl. Acad. Sci. USA* 102(20), 7233–7238 (2005).

▶40 Abramson J, Giraud M, Benoist C, Mathis D. Aire's partners in the molecular control of immunological tolerance. *Cell* 140(1), 123–135 (2010).

▶41 Pitkänen J, Vähämurto P, Krohn K, Peterson P. Subcellular localization of the autoimmune regulator protein. characterization of nuclear targeting and transcriptional activation domain. *J. Biol. Chem.* 276(22), 19597–19602 (2001).

▶42 Kalkhoven E. CBP and p300: HATs for different occasions. *Biochem. Pharmacol.* 68(6), 1145–1155 (2004).

▶43 Peterson P, Org T, Rebane A. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat. Rev. Immunol.* 8(12), 948–957 (2008).

▶44 Liiv I, Rebane A, Org T *et al.* DNA-PK contributes to the phosphorylation of *AIRE*: importance in transcriptional activity. *Biochim. Biophys. Acta* 1783(1), 74–83 (2008).

▶45 Ilmarinen T, Kangas H, Kytömaa T *et al.* Functional interaction of *AIRE* with PIAS1 in transcriptional regulation. *Mol. Immunol.* 45(7), 1847–1862 (2008).

▶46 Aloj G, Giardino G, Valentino L *et al.* Severe combined immunodeficiencies: new and old scenarios. *Int. Rev. Immunol.* 31(1), 43–65 (2012).

▶47 Oven I, Brdicková N, Kohoutek J, Vaupotic T, Narat M, Peterlin BM. AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. *Mol. Cell. Biol.* 27(24), 8815–8823 (2007).

▶48 Koh AS, Kuo AJ, Park SY *et al.* Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. *Proc. Natl. Acad. Sci. USA* 105(41), 15878–15883 (2008).

▶49 Matsumoto M. Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla. *Eur. J. Immunol.* 41(1), 12–17 (2011).

▶50 Yano M, Kuroda N, Han H *et al.* Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J. Exp. Med.* 205(12), 2827–2838 (2008).

▶51 Poliani PL, Kisand K, Marrella V *et al.* Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. *Am. J. Pathol.* 176(3), 1104–1112 (2010).

▶52 Cavadini P, Vermi W, Facchetti F *et al.* *AIRE* deficiency in thymus of 2 patients with Omenn syndrome. *J. Clin. Invest.* 115(3), 728–732 (2005).

▶53 Notarangelo L, Casanova JL, Conley ME *et al.*; International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee Meeting in Budapest, 2005. *J. Allergy Clin. Immunol.* 117(4), 883–896 (2006).

▶54 Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J. Clin. Endocrinol. Metab.* 91(8), 2843–2850 (2006).

•• **Describes the phenotypic variability of the largest series of autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) patients.**

▶55 Zlotogora J, Shapiro MS. Polyglandular autoimmune syndrome type I among Iranian Jews. *J. Med. Genet.* 29(11), 824–826 (1992).

▶56 Aaltonen J, Björses P, Sandkuijl L, Perheentupa J, Peltonen L. An autosomal locus causing autoimmune disease: autoimmune polyglandular disease type I assigned to chromosome 21. *Nat. Genet.* 8(1), 83–87 (1994).

▶57 Rosatelli MC, Meloni A, Meloni A *et al.* A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients. *Hum. Genet.* 103(4), 428–434 (1998).

▶58 Wolff AS, Erichsen MM, Meager A *et al.* Autoimmune polyendocrine syndrome type 1 in Norway: phenotypic variation, autoantibodies, and novel mutations in the autoimmune regulator gene. *J. Clin. Endocrinol. Metab.* 92(2), 595–603 (2007).

▶59 Capalbo D, Mazza C, Giordano R *et al.* Molecular background and genotype-phenotype correlation in autoimmune-polyendocrinopathy-candidiasis-ectodermal-distrophy patients from Campania and in their relatives. *J. Endocrinol. Invest.* 35(2), 169–173 (2012).

• **The study illustrates the high intrafamilial variability of APECED in subjects carrying the same mutation.**

▶60 Clemente MG, Meloni A, Obermayer-Straub P, Frau F, Manns MP, De Virgiliis S. Two cytochromes P450 are major hepatocellular autoantigens in autoimmune polyglandular syndrome type 1. *Gastroenterology* 114(2), 324–328 (1998).

▶61 Meloni A, Perniola R, Faà V, Corvaglia E, Cao A, Rosatelli MC. Delineation of the molecular defects in the *AIRE* gene in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients from Southern Italy. *J. Clin. Endocrinol. Metab.* 87(2), 841–846 (2002).

▶62 Cervato S, Mariniello B, Lazzarotto F *et al.* Evaluation of the autoimmune regulator (*AIRE*) gene mutations in a cohort of Italian patients with autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) and in their relatives. *Clin. Endocrinol.* 70(3), 421–428 (2009).

▶63 Giordano C, Modica R, Allotta ML *et al.* Autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) in Sicily: confirmation that R203X is the peculiar *AIRE* gene mutation. *J. Endocrinol. Invest.* 35(4), 384–388 (2012).

▶64 Betterle C, Greggio NA, Volpato M. Clinical review 93: autoimmune polyglandular syndrome type 1. *J. Clin. Endocrinol. Metab.* 83(4), 1049–1055 (1998).

▶65 Ahonen P, Myllärniemi S, Sipilä I, Perheentupa J. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N. Engl. J. Med.* 322(26), 1829–1836 (1990).

▶66 Meager A, Visvalingam K, Peterson P *et al.* Anti-interferon autoantibodies in autoimmune polyendocrinopathy syndrome type 1. *PLoS Med.* 3(7), e289 (2006).

▶67 Alimohammadi M, Björklund P, Hallgren A *et al.* Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. *N. Engl. J. Med.* 358(10), 1018–1028 (2008).

▶68 Betterle C, Dal Pra C, Mantero F, Zanchetta R. Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. *Endocr. Rev.* 23(3), 327–364 (2002).

▶69 Tóth B, Wolff AS, Halász Z *et al.* Novel sequence variation of *AIRE* and detection of interferon-omega antibodies in early infancy. *Clin. Endocrinol.* 72(5), 641–647 (2010).

▶70 Kisand K, Bøe Wolff AS, Podkrajsek KT *et al.* Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J. Exp. Med.* 207(2), 299–308 (2010).

▶71 Puel A, Döffinger R, Natividad A *et al.* Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J. Exp. Med.* 207(2), 291–297 (2010).

▶72 Ströbel P, Murumägi A, Klein R *et al.* Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinopathy syndrome type 1 (APS-1). *J. Pathol.* 211(5), 563–571 (2007).

▶73 Kisand K, Lilic D, Casanova JL, Peterson P, Meager A, Willcox N. Mucocutaneous candidiasis and autoimmunity against cytokines in APECED and thymoma patients: clinical and pathogenetic implications. *Eur. J. Immunol.* 41(6), 1517–1527 (2011).

•• **Discusses chronic mucocutaneous candidiasis pathogenesis and hypothesizes a novel pathogenetic mechanism for APECED on the basis of recent documented anti-Th17-related cytokines.**

▶74 Michels AW, Gottlieb PA. Autoimmune polyglandular syndromes. *Nat. Rev. Endocrinol.* 6(5), 270–277 (2010).

▶75 Halonen M, Eskelin P, Myhre AG *et al.* *AIRE* mutations and human leukocyte antigen genotypes as determinants of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy phenotype. *J. Clin. Endocrinol. Metab.* 87(6), 2568–2574 (2002).

▶76 Kekäläinen E, Tuovinen H, Joensuu J *et al.* A defect of regulatory T-cells in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J. Immunol.* 178(2), 1208–1215 (2007).

▶77 Laakso SM, Kekäläinen E, Rossi LH *et al.* IL-7 dysregulation and loss of $CD8^+$ T cell homeostasis in the monogenic human disease autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J. Immunol.* 187(4), 2023–2030 (2011).

▶78 Capalbo D, Elefante A, Spagnuolo MI *et al.* Posterior reversible encephalopathy syndrome in a child during an accelerated phase of a severe APECED phenotype due to an uncommon mutation of *AIRE*. *Clin. Endocrinol. (Oxf)* 69(3), 511–513 (2008).

▶79 Pignata C, Fiore M, de Filippo S, Cavalcanti M, Gaetaniello L, Scotese I. Apoptosis as a mechanism of peripheral blood mononuclear cell death after measles and varicella-zoster virus infections in children. *Pediatr. Res.* 43(1), 77–83 (1998).

3.3. Conclusive remarks

The abnormal functionality of the immune system leads to immunedeficiencies, genetic or acquired diseases showing predisposition to severe infections, which in certain circumstances may also cause autoimmunity and cancer. The prototype of PIDs associated with predisposition to cancer and autoimmunity are, respectively, A-T and APECED diseases.

A-T is considered a DNA repair defect syndromes, associated to degeneration of specific tissue affecting particularly the nervous and immune systems, chromosomal instability, and sensitivity to specific DNA-damaging agents. Recently, an improvement of neurological signs during short-term treatment with oral betamethasone has been reported, even though the molecular and biochemical mechanisms by which the steroid produces such effects have not yet been elucidated. A better understanding of the mechanisms of action of GCs in the brain is needed and it should open a new window of intervention in this so far non-curable disease.

It is known that the systemic autoimmunity and immunodeficiency can be strictly linked. The discovery of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms enormously contributed to our understanding of the molecular basis of human autoimmune disorders, generally and appropriately considered as multifactorial diseases. The paradigm of the genetically determined alteration of central tolerance is related to mutations of the AIRE transcription factor. This disease gave a huge amount of information on the functional and molecular events that lead to the elimination of self-reactive T-cell clones within the thymus. The elucidation that TSA are broadly expressed by mTECs also helped in understanding the

mechanism by which autoimmunity may also paradoxically be associated with an immunodeficiency status.

CHAPTER IV

"Rare Genetic Syndromes involving Immune System"

4.1. SCID-Like phenotype associated with an inhibitory autoreactive immunoglobulin

The SCIDs, irrespectively of the individual genetic form, show abnormal T cell-related functions, thus overall compromising a normal, productive immune response in all effector tasks (249). Severe impairment of T-cell function, however, may also be acquired and induced by viruses, such as the human immunodefi ciency virus (HIV) (250), but no autoreactive anti-lymphocyte antibody capable of inducing a SCID-like phenotype has been previously described.

For the first time, our group described the autoreactive anti-lymphocyte antibody capable of inducing a SCID-like phenotype in 3-year-old patient with a phenocopy of T^{low} B^{+} NK^{+} -SCID. The patient shows inhibition of normal peripheral mononuclear cell (PBMC) proliferation after stimulation with phytohemagglutinin (PHA), which could be due to inhibitory autoantibody in our patient, as a consequence of hyperimmune dysregulation.

Importantly, we have documented a novel pathogenetic mechanism due to an inhibitory anti-lymphocytic autoantibody in a SCID-like phenotype, resulting in total T-cell activation deficiency associated with autoimmunity.

The data have been published as *Case Report* on *Journal of Investigational Allergology & Clinical Immunology*, for the manuscript see below.

In our case, the clinical and histological features were compatible with a diagnosis of Sweet's syndrome. A reaction to azathioprine was suspected due to the temporal relationship between drug administration and onset of lesions and the resolution of signs and symptoms after withdrawal. Recurrence after an oral challenge confirmed the suspected diagnosis of azathioprine-induced Sweet's syndrome. In the second episode the symptoms resolved sooner, presumably due to a shorter exposure to the drug.

The first convincing case of azathioprine-induced Sweet's syndrome was reported by Stapleton in 2003. Since then, only 6 cases with a plausible link to the use of azathioprine have been described. In 2 of these, the causal relationship was not firmly established because an oral challenge was not performed and the underlying diseases (myasthenia gravis and Crohn's disease) might have been responsible for the reactions reported. The other 4 reports were associated with inflammatory bowel disease [4-7]. In all of these cases, azathioprine was strongly implicated as the causal agent as there was a well-defined temporal relationship, resolution of lesions after drug discontinuation, and a new eruption after the reintroduction of azathioprine. In none of the cases were allergy tests carried out.

To the best of our knowledge, there are no reports of azathioprine-induced Sweet's syndrome associated with microscopic polyangiitis in the literature.

The pathogenesis of Sweet's syndrome is unknown. It has been defined as a type-III hypersensitivity phenomenon, although there is new evidence that suggests the involvement of other mechanisms, including elevated granulocyte colony-stimulating factor [8], associations with determined histocompatibility (Bw54)[9], and a possible role of antineutrophil cytoplasmic antibodies in the activation of neutrophils [10].

We emphasize the importance of evaluating azathioprine as a possible, though uncommon, cause of Sweet´s syndrome and of replacing it with a non-purine analog treatment if the involvement of azathioprine is confirmed.

References

1. Sweet RD. An acute febrile neutrophilic dermatosis. Br J Dermatol. 1964; 76:349-56.
2. Thompson DF, Montarella KE. Drug-induced Sweet's syndrome. Ann Pharmacother. 2007; 41:802-11.
3. Rokea A, el-Azhary MD. Azathioprine: current status and future considerations. Int J Dermatol. 2003; 42:335-41.
4. Paoluzi OA, Crispino P, Amantea A, Pica R, Iacopini F, Consolazio A, Di Palma V, Rivera M, Paoluzi P. Diffuse febrile dermatosis in a patient with active ulcerative colitis under treatment with steroids and azathioprine: a case of Sweet's syndrome. Case report and review of literature. Dig Liver Dis. 2004; 36:361-6.
5. El-Azhary RA, Brunner KL, Gibson LE. Sweet syndrome as a manifestation of azathioprine hypersensitivity. Mayo Clin Proc. 2008; 83:1026-30.
6. Treton X, Joly F, Alves A, Panis Y, Bouhnik Y. Azathioprine-induced Sweet's syndrome in Crohn's disease. Inflamm Bowel Dis. 2008; 14:1757-8.
7. Yiasemides E, Thom G. Azathioprine hypersensitivity presenting as a neutrophilic dermatosis in a man with ulcerative colitis. Australas J Dermatol. 2009; 50:48-51.
8. Kawakami T, Ohashi S, Kawa Y, Takahama H, Ito M, Soma Y, Mizoguchi M. Elevated serum granulocyte colony-stimulating factor levels in patients with active phase of sweet syndrome and patients with active behcet disease: implication in neutrophil apoptosis dysfunction. Arch Dermatol. 2004; 140:570-4.
9. Mizoguchi M, Matsuki K, Mochizuki M, Watanabe R, Ogawa K, Harada S, Hino H, Amagi M, Juji T. Human leukocyte antigen in Sweet's syndrome and its relationship to Behçet's disease. Arch Dermatol. 1988; 124:1069-73.
10. Darné S, Natarajan S, Blasdale C. Do antineutrophil cytoplasmic antibodies (ANCA) play a key role in neutrophilic dermatoses? A case of propylthiouracil-induced neutrophilic dermatosis with positive perinuclear ANCA. Clin Exp Dermatol. 2010; 35:406-8.

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SCID-Like Phenotype Associated With an Inhibitory Autoreactive Immunoglobulin

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Key words:Severe combined immunodeficiency. Autoantibody. SCID-like phenotype. T-cell activation defect. Serum inhibitory factor.

Palabras clave: Inmunodeficiencia combinada grave. Autoanticuerpo. Fenotipo IDCG. Defecto de activación de linfocitos T. Factor inhibidor sérico.

Severe combined immunodeficiency (SCID) includes a number of distinct entities that share several clinical hallmarks such as life-threatening infections and intractable diarrhea [1], rapidly leading to growth failure and malnutrition. The clinical course is severe and patients usually die before the second year of life unless a stem cell transplantation is performed. SCID is currently classified into several groups based on the presence or absence of the major lymphocyte cellular components T, B or natural killer (NK) cells, with each group being suggestive of 1 or more genetic causes [1]. However, irrespectively of the individual genetic form, T cell–related functions are constantly abnormal, thus overall compromising a normal, productive

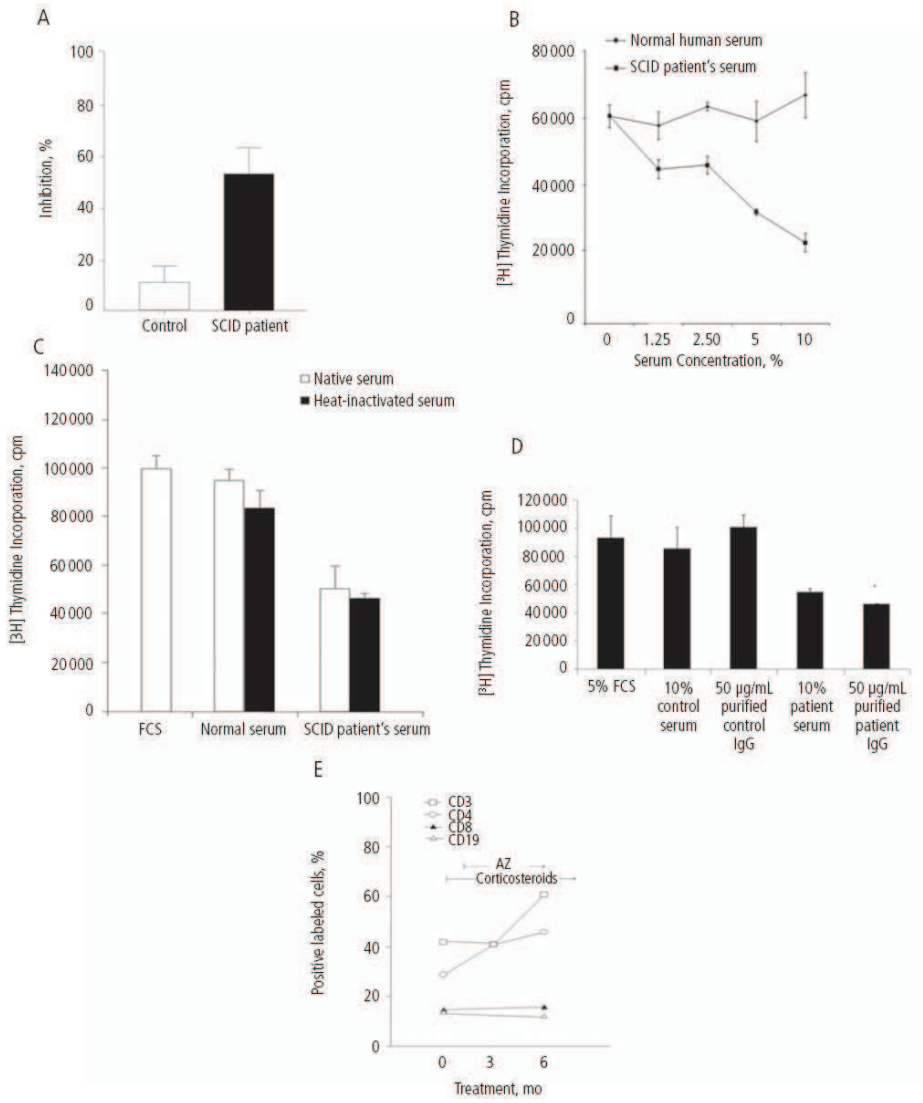

Figure. A, Inhibition (%) of normal peripheral mononuclear cell (PBMC) proliferation after stimulation with phytohemagglutinin (PHA, 8 µg/mL). B, [3H] thymidine incorporation by normal PBMCs stimulated with PHA, (8 µg/mL) and incubated with scalar concentrations (0%, 1.25%, 2.5%, 5%, and 10%) of normal human serum or serum from a patient with severe combined immunodeficiency (SCID). Each point represents the mean (SD), (n=3). C, Effect of native and heat-inactivated serum on proliferative response by normal PBMCs stimulated with PHA (8 µg/mL). Values are expressed as mean (SD) (n=3). D, Inhibition by patient's immunoglobulin (Ig) G of proliferative response by normal PBMCs stimulated with PHA (8 µg/mL). Negative control: fetal calf serum (FCS). Bars show means (SD). *Statistically significant difference ($P<.05$) compared to cultures containing purified control IgG fraction. E, Increase in the percentage of major lymphocyte subsets in patient during treatment with azathioprine (AZ) and corticosteroids. The horizontal lines indicate the period of immunosuppressive treatment with AZ (dotted line) and corticosteroids (solid line). Cpm indicates counts per minute.

immune response in all effector tasks [2]. Severe impairment of T-cell function, however, may also be acquired and induced by viruses, such as the human immunodeficiency virus (HIV) [3]. To date, no autoreactive anti-lymphocyte antibody capable of inducing a SCID-like phenotype has been described.

We report on a 3-year-old patient with a phenocopy of T_{low} B^+ NK^+ SCID. The patient was born at 42 weeks of gestation to healthy, unrelated parents. At 6 months of age the child was hospitalized because of chronic diarrhea, dystrophic features, and febrile seizures. At 8 months of age, the immunological evaluation revealed decreased immunoglobulin (Ig) G serum levels (<47 mg/dL) and normal IgA and IgM. Moreover, the patient had autoimmune hemolytic anemia. At the time of the study, lymphocytes were 3×10^9/L, with 34.5% of $CD3^+$ cells, 25.5% of $CD4^+$ cells, 15.3% of $CD8^+$ cells, 4% of $CD19^+$ cells, and 15% of $CD56^+CD3^-$ cells. Severe lymphocyte functional impairment, in the absence of infection by HIV or any other viruses, was noted. The proliferation assays using phytohemagglutinin (PHA, 8 μg/mL) was performed as previously described [4]. The patient's peripheral blood mononuclear cells (PBMCs) exhibited absent proliferative response to PHA (mean [SD] of 768 [61] counts per minute [cpm] vs 104 649 [21 743] cpm in controls). Over the 3-year follow-up, the patient's PBMC proliferative response ranged between 300 and 8095 cpm. To identify a potential inhibitory factor, the patient's serum was added to PBMCs from 5 healthy controls. A significantly higher inhibitory effect was noted in the patient's serum (53% [10%]) compared to that of the controls (12% [5%]) (Figure A). To define the potency of the inhibitory effect, scalar doses (0%, 1.25%, 2.5%, 5%, and 10%) of the patient's serum and the normal serum were used to produce a dose-response curve. A linear increase in inhibition was observed, with maximum inhibition being reached at the 10% concentration (23 729 [2701] cpm vs 67 050 [6638] cpm in the presence of the control serum) (Figure B). Serum heat inactivation did not abolish the inhibitory effect on the proliferative response to PHA in the control PBMCs, in that the inhibition was 47% (compared to 51% for native serum) ($P>.05$) (Figure C), thus ruling out a role of the complement in the phenomenon.

To evaluate whether the inhibitory effect in the patient's serum was attributable to an anti-lymphocyte autoantibody, affinity-purified IgG from both the patient's and the control serum was tested for the inhibitory property. Significant inhibition of the proliferative response of normal PHA-stimulated PBMCs was seen in the former but not in the latter case (46 204 [473] cpm vs 100 778 [8988] cpm; $P<.05$). The inhibition in the presence of the patient's IgG fraction was comparable to that observed with the patient's serum ($P>.05$) (Figure D).

Thereafter, a progressive decline in $CD4^+$ cells, resulting in a typical lymphocytopenic (0.5×10^9/L) T^- B^+ NK^+ form of SCID, was observed. A similar phenotype is generally due to an impairment in the T-cell differentiation process, resulting in a severe reduction in peripheral T-cell pool size associated with molecular alterations of genes implicated in T-cell ontogeny and function [5]. The prototype of severe T-cell defects, in which NK cells are also often compromised, is related to mutations of the *IL-2Rγ* gene. However, in our case, such gene alterations were ruled out. Two episodes of bronchopneumonia and an interstitial pneumopathy occurred despite intravenous IgG replacement therapy and antibiotic treatment. Autoreactive antibodies toward smooth muscle and red and white blood cells were detected. The patient also developed severely progressive active autoimmune hepatitis, which was diagnosed according to the scoring system established by the International Autoimmune Hepatitis group [6] and treated with azathioprine (1.5 mg/kg/d) and corticosteroids. Diarrhea, autoimmunity, and liver disorders are usually described in relation to T^+ oligoclonal B^-Omenn syndrome [7] and T_{low} B^+ $IL\text{-}7R\alpha$ deficiency [8]. Even though these genetic defects were not ruled out, since at the time of evaluation this information was not available, the clinical and immunological features in our patient are quite different from those seen in these syndromes. At 4 years of age the patient died of disseminated interstitial pneumopathy while the search for an HLA-matched donor was still underway. During this period a paradoxical effect of immunosuppression on cell subsets was noted in that, as depicted in Figure E, there was an increase in $CD3^+$ cells from 42.0% to 60.9% and in $CD4^+$ cells from 28.0% to 46.3%. By contrast, $CD19^+$ and $CD8^+$ cells did not change significantly.

Our data indicate a direct role of the antibody as a negative regulator of T-cell function. However, it is also possible that the inhibitory autoantibody in our patient was the consequence of hyperimmune dysregulation rather than of the T-cell defect, whose genetic alteration still remains to be identified. A similar serum inhibitory effect has also been described in epilepsy, leading to moderate T-cell dysfunction associated with impairment of other immunological cell activities and a decrease in the C4 complement component [9]. However, this functional lymphocyte defect was not as severe as that reported in our patient. Although the functional defect observed in our case may theoretically be related to viral-induced anergy [10], no viral infection was documented and the functional defect was completely differently from that observed in viral-induced anergy.

In conclusion, we have documented a novel pathogenetic mechanism due to an inhibitory anti-lymphocytic autoantibody in a SCID-like phenotype, resulting in total T-cell activation deficiency associated with autoimmunity. This complex phenotype represents a phenocopy of the congenital forms of SCID.

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References

1. Notarangelo LD. Primary immunodeficiencies. J Allergy Clin Immunol. 2010;125:S182-94.
2. Fischer A. Severe combined immunodeficiencies (SCID). Clin Exp Immunol. 2000;122:143-9.
3. Gu XXJ, Yue FY, Kovacs CM, Ostrowski MA. The role of cytokines which signal through the common g chain cytokine receptor in the reversal of HIV specific CD4+ and CD8+ T cell anergy. Plos one. 2007;2:e300.

4. Pignata C, Troncone R, Monaco G, Ciriaco M, Farris E, Carminati G, Auricchio S. Impaired suppressor activity in children affected by coeliac disease. Gut. 1985;26:285-90.
5. Kalman L, Lindegren ML, Kobrynski L, Vogt R, Hannon H, Howard JT, Buckley R. Mutions in genes required for T-cell development: Il7R, CD45, Il2RG, JAK3, RAG1, RAG2, ARTEMIS, and ADA and severe combined immunodeficiency: HuGE review. Genet Med. 2004;6:16-26.
6. Álvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WGE, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston ALWF, Fainboim L, Heatcotre J, Homberg JC, Hoofnagle H, Kakumu S, Krawitt EL, Mackay IR, MacSween RNM, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Mieli-Vergani G, Nakanuma Y, Nishioka M, Penner E, Porta G, Portmann BC, Reed WD, Rodes J, Schalm SW, Scheuer PJ, Schrumpf E, Seki T, Toda G, Tsuji T, Tygstrup N, Vergani D, Zeniya M. International autoimmune hepatitis group report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol. 1999;31:929-38.
7. Villa A, Notarangelo L, Roifman CM. Omenn syndrome: Inflammation in leaky severe combined immunodeficiency. J Allergy Clin Immunol. 2008;122:1082-6.
8. Giliani S, Mori L, De Saint Basile G, F. LD, Rodriguez-Perez C, Forino C, Mazzolari E, Dupuis S, Elhasid R, Kessel A, Galambrun C, Gil J, Fischer A, Etzioni A, Notarangelo L. Interleukin-7 receptor a (IL-7Ra) deficiency: cellular and molecular bases. Analysis of clinical, immunological, and molecular features in 16 novel patients. Immunol Rev. 2005;203:110-26.
9. Montelli TCB, Soares AMVC, Peracoli MTS. Immunologic aspects of West Syndrome and evidence of plasma inhibitory effects on T cell function. Arq Neuropsiquiatr. 2003;61:731-7.
10. Pignata C, Fiore M, De Filippo S, Cavalcanti M, Gaetaniello L, Scotese I. Apoptosis as a mechanism of peripheral blood mononuclear cell death following measles and varicella-zoster virus infections in children. Ped Res. 1998;43:77-83.

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Bronchospasm Induced Selectively by Paracetamol

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Key words: Paracetamol. Bronchospasm. Neurogenic inflammation.

Palabras clave: Paracetamol. Broncoespamo. Inflamación neurogénica.

It is well known that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can trigger bronchospasm in susceptible individuals as a result of inhibitory cyclooxygenase (COX) activity. Paracetamol (acetaminophen) is a weak inhibitor of this pathway, but in some patients with NSAID idiosyncrasy, high doses of paracetamol can also provoke bronchospasm.

Although in recent years several epidemiological studies have reported an increased risk of asthma in relation to paracetamol use [1,2], to our knowledge, no cases of selective bronchospasm induced by paracetamol have been published.

We report the case of a patient who developed bronchospasm after paracetamol intake.

A 19-year-old woman was referred to our hospital because she had experienced 8 to 10 episodes of dyspnea within 10 minutes of taking paracetamol over the previous 3 years; this had not occurred with any other NSAIDs (metamizole, ibuprofen). The patient also had a clinical history of rhinitis associated with contact with cats and bronchospasm after exercise.

Skin prick tests to aeroallergens showed positivity for *Alternaria alternata* and for *Olea europaea* pollen. Total immunoglobulin (Ig) E was 41 kU/L and specific IgE to *Alternaria alternata* and cat dander was 3.47 kU/L and 0.23 kU/L, respectively. Spirometry showed mild ventilatory alteration and the bronchodilator response was positive (>12% increase in forced expiratory response in the first second [FEV_1]).

With a diagnosis of rhinitis and asthma with *Alternaria alternata* sensitization, we performed a study with paracetamol with the patient's consent. The prick test (10 mg/mL) and intradermal test (1 mg/mL) to paracetamol were negative. An oral challenge test with paracetamol (100-250-500 mg) and acetylsalicylic acid (ASA) (500 mg) was carried out on different days. Spirometry was performed at baseline and 10 minutes after the intake of placebo and the above-mentioned drugs. The challenge was considered positive when there was a decrease of over 12% in FEV_1 compared to baseline. Salbutamol inhalation was used to evaluate bronchial reversibility after the paracetamol challenge.

Ten minutes after the administration of 500 mg of paracetamol the patient presented bronchospasm and a significant decrease in spirometric values, which returned to baseline after salbutamol inhalation. There were no other systemic or cutaneous symptoms. The administration of 500 mg of ASA did not induce any changes (Table).

To our knowledge this is the first report that confirms the induction of bronchospasm by paracetamol without the involvement of other NSAIDs.

The mechanism by which paracetamol induces bronchospasm in our patient is unclear.

NSAIDs have the ability to induce bronchospasm by inhibiting COX-1, and paracetamol is a weak inhibitor of this enzyme. The fact that high doses of ASA did not induce bronchospasm in the patient makes it highly unlikely that paracetamol acts in this pathway. We also failed to demonstrate specific IgE by means of skin tests.

While a neurogenic mechanism has been implicated as responsible for airway inflammation [3], some authors, based on experimental studies, have more recently suggested that the activation of specific receptors expressed on sensorial neurons could induce inflammation in the airways. In this

4.2. A novel mutation of IL2Rβ associated to an atypical Immunedeficiency

Interleukin-12 (IL-12) is involved in cellular immune responses against intracellular pathogens by promoting the generation of T naive in T helper 1 (Th1) cells and by increasing interferon-gamma (IFN-gamma) production from T and natural killer (NK) cells. A defective induction of a Th1 response may lead to a higher risk of infections, and, in particular, infections due to typical and atypical Mycobacteria. We report on the case of a girl with suffering from recurrent bronchopneumonia associated with very high serum IgE levels, who exhibited a profound impairment of the Th1 generation associated with a novel mutation in the exon 5 of the IL-12R β1 gene (R156H). Our data suggest that in children with severe and recurrent infections, even in the absence of a mycobacterial infection, functional and/or genetic alterations of the molecular mechanisms governing Th1/Th2 homeostasis might be responsible for an atypical immunodeficiency and, therefore, should be investigated in these patients.

The data is being published as *Clinical Report* on *American Journal of Medical Genetics*, for the manuscript see below.

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CASE REPORT Open Access

Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and very high IgE levels

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Abstract

Interleukin-12 (IL-12) is involved in cellular immune responses against intracellular pathogens by promoting the generation of T naive in T helper 1 (Th1) cells and by increasing interferon-gamma (IFN-gamma) production from T and natural killer (NK) cells. A defective induction of a Th1 response may lead to a higher risk of infections, and, in particular, infections due to typical and atypical *Mycobacteria*. We report on the case of a girl with suffering from recurrent bronchopneumonia associated with very high serum IgE levels, who exhibited a profound impairment of the Th1 generation associated with a novel mutation in the exon 5 of the IL-12R β1 gene (R156H). Our data suggest that in children with severe and recurrent infections, even in the absence of a mycobacterial infection, functional and/or genetic alterations of the molecular mechanisms governing Th1/Th2 homeostasis might be responsible for an atypical immunodeficiency and, therefore, should be investigated in these patients.

Keywords: Immunodeficiency, IL-12/IL-12 receptor, Recurrent pneumonia

Background

Primary congenital immunodeficiencies encompass a wide spectrum of distinct clinical entities, which differ in either pathogenetic mechanism or clinical features. Recently, several novel syndromes with unusual phenotypes have been described [1,2]. However, in a number of patients suffering from severe and sometimes life-threatening infections, in which an immunological disorder is suspected, the underlying genetic defect responsible for the immunodeficiency still remains to be elucidated [3]. Recently, a higher susceptibility to intracellular pathogens and, in particular, atypical mycobacterial and salmonella infections has been described in patients with genetic alterations of the IL-12 receptor (IL-12R) [4-9]. IL-12 stimulates cellular immune responses against intracellular pathogens by promoting the generation of T naive in T helper 1 (Th1) cells and by increasing interferon-gamma (IFN-gamma) production from T and natural killer (NK) cells. Induction of a Th1 response and cell cycle progression mostly relies on the expression of a high affinity IL-12R, consisting of β1 and β2 chains [10-15]. A few genetic alterations of β1 chain have already been reported in patients suffering of mycobacterial infections [5,6].

Case presentation

A 8-year-old girl was referred to the our Department because of a history of recurrent pneumonia (4 episodes over 2 years). At the age of 4 years and 8 months she had suffered from the first episode of middle lobar bronchopneumonia requiring hospitalization. In that occasion the total IgE serum levels were 3350 kU/l. One month later, she was hospitalized for a second bronchopneumonia episode interesting both lungs followed by persistent cough for more than a month. These episodes were responsive to antibiotic therapy. Subsequently, she had suffered from 2 additional bronchopneumonia episodes in distinct lung area, successfully treated with parenteral antibiotic therapy. Conventional x-ray and high resolution computed tomography of the chest revealed multiple focal consolidations in both lungs, confirmed

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by magnetic resonance imaging [16]. Acid resistant bacillus was not found in the sputum examination. In one occasion, *Haemophilus influenzae* was isolated on sputum culture. Weight and height growth was in the normal range. No infections in other organs were reported. The routine immunological evaluation revealed normal IgG and IgA, but very high serum IgE levels (> 2000 kU/l), confirmed in several occasions during the 2 years follow-up. Specific IgE toward Dermatophagoides farinae and pteronyss, olive, herb vitriol and Parietaria judaica were detected. Prick test were positive (ponf > 0.3 × 0.4 cm) for Dermatophagoides farinae and pteronyss, hair of dog, Parietaria and olive, thus confirming a multiple sensitivity. The patient showed a proper antibody specific response as demonstrated by the presence of IgG antibody serum levels, tested by immuno-enzymatic method, against B-hepatitis, parotitis and German measles viruses. Serum IgG, IgA e IgM levels were always in the normal range. The immunophenotype valuation revealed normal number and percentage of the lymphocyte subpopulations studied (Table 1).

The patient's family history was notable for the presence of allergic disorders in both lineages. In particular, her mother and grandmother had a history of allergic rhinitis, while her father had urticaria. A maternal aunt died at 2 years of age by whooping-cough and a maternal uncle died at 16 months by a severe not better specified respiratory infection. A paternal aunt and her daughter had a history of allergic rhinitis.

We first determined the proliferative response of PBMC to CD3 cross-linking, that mimics in vivo antigen exposure, performed at optimal (1 ng/ml) or suboptimal (0.3 ng/ml) antibody concentration. The proliferation at the maximal dosage was significantly lower in the patient than in the controls (mean ± SE were: 800 ± 68 cpm and 29500 ± 3000 cpm in the proband and controls, respectively). Since a proper immune response to pathogens requires a Th1 induction and this process determines up-regulation of the expression of the IL-12R β2 chain, we evaluated mRNA expression of this molecule after mitogen stimulation of PBMC in vitro. No expression of IL-12R β2 transcript was found in patient's cells (Figure 1A), differently from the controls. IL-12R β1 was expressed at normal levels (Figure 1B).

At molecular level, gene sequencing of IL-12R β2 gene revealed a missense mutation (G to A) at nucleotide 531 in the exon 5 in heterozygosity, resulting in the substitution of arginine (CGT) with histidine (CAT) in the extracellular domain of the receptor at the same aminoacid position 156 (designed R156H) (Figure 2). The mutation was not a polymorphism since was not found in 100 chromosomes from unrelated individuals. This G to A transition creates a new restriction site for NdeI enzyme (data not shown).

Table 1 Immunonological parameters

Lymphocyte subpopulations	%	n/mm³
CD3	77	2956
CD3DR	4.2	161.28
CD4	39.3	1509
CD8	30.2	1159
CD19	11.4	437.76
CD56	3.4	130.56
CD4-CD8-TCR α/β+	2.3	88.32
CD4-CD8-TCR g/d+	2.2	84.48
Specific antibody responses	**IgG**	**IgM**
B-hepatitis virus	Present	Absent
Parotidis virus	Present	Absent
German measles virus	Present	Absent
Proliferative response to mitogens stimulation	**Patient (mean ± SE)**	**Control (mean ± SE)**
PMA + iono	22458 ± 11013	32159 ± 27858
CD3 X-L	800 ± 68	29500 ± 3000

Discussion

The case here reported indicates that alterations of the induction of a proper Th1 response may be associated with an atypical immunodeficiency characterized by high susceptibility to infections. The functional response of lymphocytes to IL-12 depends on the expression of a high affinity IL-12 receptor on Th1 and NK cells. The high affinity receptor for IL-12 consists of two subunits,

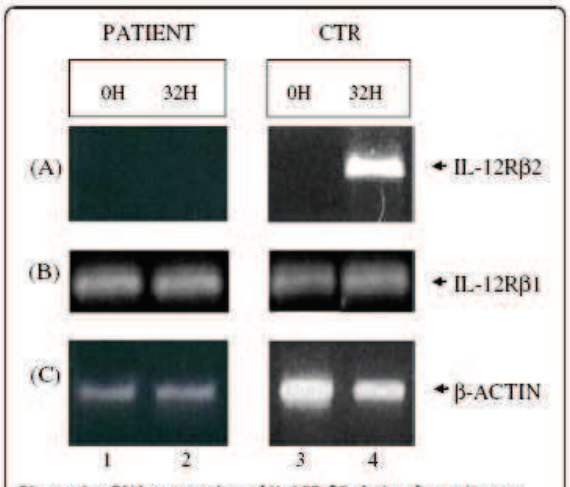

Figure 1 mRNA expression of IL-12R β2 chain after mitogen stimulation of PBMC in vitro. (A) No expression of IL-12R β2 transcript was found in patient's cells, differently from the controls. (B) IL-12R β1 was expressed at normal levels.

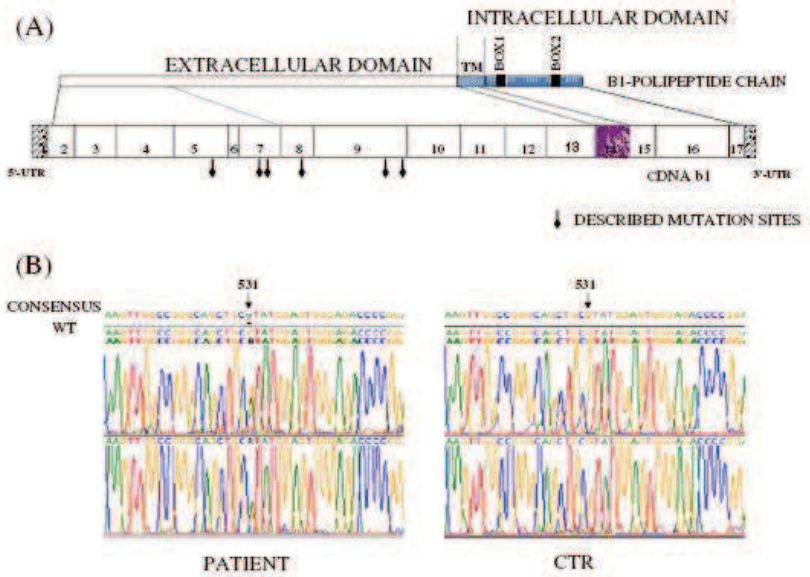

Figure 2 gene sequencing of IL-12R β2 gene. At molecular level, gene sequencing of IL-12R β2 gene revealed a missense mutation (G to A) at nucleotide 531 in the exon 5 in heterozygosity, resulting in the substitution of arginine (CGT) with histidine (CAT) in the extracellular domain of the receptor at the same aminoacid position 156 (designed R156H).

β1 and β2, closely related to the cytokine receptor glycoprotein (gp) 130 [11,17]. The complete IL-12R is thought to be associated with the development, being expressed on human naive T cells during differentiation to Th1 but not to Th2. Therefore, the expression of these molecules is generally considered as a marker of Th1 dominated response [11-13,16]. Th1 cells produce IFN-gamma and IL-2 and, predominantly, promote cell mediate immune responses against intracellular pathogens [18,19]. In a previous study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE levels, suggestive of an impaired Th1 induction [20]. A defective induction of a Th1 response in patients may lead to a higher risk of infections, thus worsening the overall outcome of patients with very high IgE levels. In the case herein described a genetic alteration of the IL-12R β1 has been found in heterozygosity. Whether this alteration is really responsible for the phenotype remains to be definitively demonstrated with further molecular and functional studies. However, it should be noted that patients with homozygous alterations of the same gene have already been reported, being affected with a more severe clinical phenotype and selective susceptibility to mycobacterial infections [5,6]. Based on this clinical observation, we suggest that a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help recognize novel clinical phenotypes of atypical immunodeficiencies.

Consent

Written informed consent was obtained from the parents of the patient for publication of this Case report and any accompanying images.

Abbreviations

IL-12: Interleukin-12; Th1: T helper 1; IFN-gamma: Interferon-gamma; NK: Natural Killer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LP has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. GG has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. FS has made substantial contributions to conception and design, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. RR and AF has made substantial contributions to acquisition of data, has been involved in drafting the manuscript, and has given final approval of the version to be published. SM has made substantial contributions to acquisition of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. MVU has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. CP has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in drafting the manuscript and revising it critically for important intellectual content, and has given final approval of the version to be published.

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References

1. Palamaro L, Vigliano I, Giardino G, Cirillo E, Aloj G, Romano R, Pignata C: SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. *J Investig Allergol Clin Immunol* 2012, 22:67–70.
2. De Vries E, Alvarez Cardona A, Abdul Latiff AH, Badolato R, Brodszki N, Cant AJ, Carbone J, Casper JT, Ciznar P, Cochino AV, Conley ME, Derfalvi B, Driessen GJ, Elfeky R, Espanol T, Gilmour K, Gueseva MN, Haverkamp MH, Helminen M, Honig M, Kanariou MG, Kirschfink M, Klein C, Kuijpers TW, Kutukculer N, Martire B, Meyts I, Niehues T, Pignata C, Reda SM, Renner ED, Rezaei N, Rizzi M, Sampaio Lainz MA, Sargur RB, Sediva A, Seidel MG, Seneviratne SL, Soler-Palacin P, Tommasini A, Warnatz K: Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. *Clin Exp Immunol* 2012, 167:108–119.
3. Aloj G, Giardino G, Valentino L, Maio F, Gallo V, Esposito T, Naddei R, Cirillo E, Pignata C: Severe combined immunodeficiences: new and old scenarios. *Int Rev Immunol* 2012, 31:43–65.
4. Aytekin C, Dogu F, Tuygun N, Tanir G, Guloglu D, Boisson-Dupuis S, Bustamante J, Feinberg J, Casanova JL, Ikinciogullari A: Bacille Calmette-Guèrin lymphadenitis and recurrent oral candidiasis in an infant with a new mutation leading to interleukin-12 receptor beta-1 deficiency. *J Invest Allergol Clin Immunol* 2011, 21:401–404.
5. Pedraza-Sanchez S, Herrera-Barrios MT, Aldana-Vergara R, Neumann-Ordoez M, Gonzalez-Hernandez Y, Sada-Diaz E, de Beaucoudrey L, Casanova JL, Torres-Rojas M: Bacille Calmette-Guèrin infection and disease with fatal outcome associated with a point mutation in the interleukin-12/interleukin-23 receptor beta-1 chain in two Mexican families. *Int J Infect Dis* 2010, 14:e256–e260.
6. Shah P, El-Maaytah M, Jerjes W, Upile T, Ayliffe P: Interleukin 12 receptor beta1 chain deficiency in a child with disseminated tuberculosis: a case report. *J Oral Maxillofac Surg* 2010, 68:909–911.
7. Granados EL, Porpiglia AS, Hogan MB, Matamoros N, Krasovec S, Pignata C, Smith CE, Hammarstrom L, Bjorkander J, Belohradsky BH, Fontan Casariego G, Garcia Rodriguez MC, Conley ME: Clinical and molecular analysis of patients with defects in micro heavy chain gene. *J Clin Invest* 2002, 110:1029–1035.
8. Altare F, Durandy A, Lammas D, Emile JF, Lamhamedi S, Le Deist F, Drysdale P, Jouanguy E, Doffinger R, Bernaudin F, Jeppsson O, Gollob JA, Meinl E, Segal AW, Fischer A, Kumararatne D, Casanova JL: Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science* 1998, 280:1432–1435.
9. Sakai T, Matsuoka M, Aoki M, Nosaka K, Mitsuya H: Missense mutation of the interleukin-12 receptor beta1 chain-encoding gene is associated with impaired immunity against Mycobacterium avium complex infection. *Blood* 2001, 97:2688–2694.
10. Presky DH, Yang H, Minetti LJ, Chua AO, Nabavi N, Wu CY, Gately MK, Gubler U: A functional interleukin 12 receptor complex is composed of two β-type cytokine receptor subunits. *Proc Natl Acad Sci USA* 1996, 93:14002–14007.
11. Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U, Sinigaglia F: Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J Exp Med* 1997, 185:825–831.
12. Szabo SJ, Dighe AS, Gubler U, Murphy KM: Regulation of the interleukin (IL)-12Rβ2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med* 1997, 185:817–824.
13. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH: The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 1998, 16:495–521.
14. Amorosi S, Russo I, Amodio G, Garbi C, Vitiello L, Palamaro L, Adriani M, Vigliano I, Pignata C: The cellular amount of the common γ-chain influences spontaneous or induced cell proliferation. *J Immunol* 2009, 182:3304–3309.
15. Vigliano I, Palamaro L, Bianchino G, Fusco A, Vitiello L, Grieco V, Romano R, Salvatore M, Pignata C: Role of the common g chain in cell cycle progression of human malignant cell lines. *Int Immunol* 2012, 24:159–167.
16. Montella S, Maglione M, Bruzzese D, Mollica C, Pignata C, Aloj G, Manna A, Esposito A, Mirra V, Santamaria F: Magnetic resonance imaging is an accurate and reliable method to evaluate non-cystic fibrosis paediatric lung disease. *Respirology* 2012, 17:87–91.
17. Presky DH, Minetti LJ, Gillessen S, Wilkinson VL, Wu CY, Gubler U, Chizzonite R, Gately MK: Analysis of the multiple interactions between IL-12 and the high affinity IL-12 receptor complex. *J Immunol* 1998, 160:2174–2179.
18. Santoni D, Pedicini M, Castiglione F: Implementation of a regulatory gene network to simulate the TH1/2 differentiation in an agent-based model of hypersensitivity reactions. *Bioinformatics* 2008, 24:1374–1380.
19. Finkelman FD, Vercelli D: Advances in asthma, allergy mechanisms, and genetics in 2006. *J Allergy Clin Immunol* 2007, 120:544–550.
20. 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* 2010, 265:74–79.

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4.3. De novo 13q12.3q14.11 deletion involving BRCA2 gene in a patient with AT-like phenotype.

Deletion of the long arm of chromosome 13 is a rare condition characterized by a wide range of clinical features, including developmental delay and intellectual disability, growth retardation with microcephaly, hypotonia, trigonocephaly, facial dysmorphism, limb defects such as hypoplastic or absent thumbs and anogenital anomalies (251). The clinical phenotype varies according to the location and size of the deletion. Involvement of the critical band 13q32 causes three major phenotypes (252). Distal deletions are closely associated with the more severe phenotypes, whereas proximal deletions cause fewer anomalies, but include retinoblastoma (253). Alterations of the ZIC2 gene, which maps on the critical 13q32 region, have been associated with holoprosencephaly (HPE) (254).

Our group report on an atypical syndrome, due to a *de novo* deletion of chromosome 13, which leads to a novel clinical phenotype characterized by immunodeficiency with elevated IgM levels, mild and transient cerebellar ataxia, increased frequency of chromosomal breaks, telangiectasia and freckles associated with microcephaly, developmental delay, facial dysmorphisms, skeletal anomalies, and spontaneous fractures. Array-comparative genomic hybridization (CGH) defined a 12Mb deletion on chromosome 13q12.3-q14.11, which includes the BRCA2 gene.

The data is being published as *Clinical Report* on *American Journal of Medical Genetics*, for the manuscript see below.

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

De novo 13q12.3–q14.11 Deletion Involving *BRCA2* Gene in a Patient With Developmental Delay, Elevated IgM Levels, Transient Ataxia, and Cerebellar Hypoplasia, Mimicking an A-T like Phenotype

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We report on a child with a de novo deletion of approximately 12 Mb detected through array comparative genomic hybridization (CGH). The deletion involved chromosome bands 13q12.3–13q14.11 and determined the loss of ≥50 genes. A second deletion on chromosome 12p11.3p11.22 of 43–167 kb, including about 12 genes, was unlikely of clinical relevance because inherited from the asymptomatic father. The child had developmental delay, dysmorphisms, and many features reminiscent of ataxia-telangiectasia (A-T), as cerebellar ataxia, oculocutaneus telangiectasia, and recurrent upper airway infections. Atraumatic fractures of the metatarsus were noted. Moreover, this is a rare case of 13q deletion syndrome associated with peripheral blood white cells radiosensitivity to bleomycin, reminiscent of what previously reported on X-ray hypersensitivity of fibroblasts from patients with alterations of this chromosome. The immunological evaluation revealed increased IgM serum levels and a low proliferative response to mitogens, PHA, and CD3 cross-linking (CD3 XL). After 12 years of age only a mild dysmetria persisted, while the proliferative response to mitogens became normal by 9 years of age.

Key words: 13q deletion; array-CGH; rare genetic syndromes; psychomotor retardation; hypotonia; radiosensitivity; cerebellar hypoplasia

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Am J Med Genet Part A 158A:2571–2576.

INTRODUCTION

Deletion of the long arm of chromosome 13 is a rare condition characterized by a wide range of clinical features, including developmental delay and intellectual disability, growth retardation with microcephaly, hypotonia, trigonocephaly, facial dysmorphism, limb defects such as hypoplastic or absent thumbs and anogenital anomalies [Allderdice et al., 1969; Grace et al., 1971]. The clinical phenotype varies according to the location and size of the deletion. Involvement of the critical band 13q32 causes three major

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phenotypes [Brown et al., 1995]. Distal deletions are closely associated with the more severe phenotypes, whereas proximal deletions cause fewer anomalies, but include retinoblastoma [Kivelä et al., 2003]. Alterations of the *ZIC2* gene, which maps on the critical 13q32 region, have been associated with holoprosencephaly (HPE)[Brown et al., 2001].

We report on a novel clinical phenotype characterized by immunodeficiency with elevated IgM levels, mild and transient cerebellar ataxia, increased frequency of chromosomal breaks, telangiectasia and freckles associated with microcephaly, developmental delay, facial dysmorphisms, skeletal anomalies, and spontaneous fractures. Array-comparative genomic hybridization (CGH) defined a 12 Mb deletion on chromosome 13q12.3–q14.11, which includes the *BRCA2* gene.

CLINICAL REPORT

The proband is a 17-year-old Caucasian male referred to our Immunodeficiency Center at the age of 3.3 years because of the presence of recurrent upper airway infections, mild ataxia, cutaneous telangiectasia, and developmental delay. He was the second child of three healthy children of nonconsanguineous parents. The family history was unremarkable. He was born at term after an uncomplicated pregnancy by cesarean. His birth weight was 2.5 kg. Fetal movements were reported as normal. A developmental delay was observed since the first months of life. The child was able to sit up and walk at 14 and 18 months, respectively. At the age of 27 months, he could pronounce 10 words. On 20 months of age, his neurological exam showed mild dysmetria and gait ataxia, muscle hypotonia, trunk titubation, and developmental delay. Brain CT obtained at 24 months of age revealed moderate enlargement of cisterna magna ventricular system due to cerebellar vermis hypoplasia. During the follow-up, brain magnetic resonance imaging (MRI), performed at 10 years of age, confirmed the moderate hypoplasia of the caudal part of the cerebellar vermis with dilatation of adjacent cerebrospinal fluid spaces. Electroencephalography was normal.

At 3.3 years of age, the physical examination revealed a height of 94 cm (25–50th centile), weight of 12.5 kg (10th centile), and head circumference of 48.1 cm (<5th centile). Dysmorphic features included long face with sparse hair, hypotelorism, dental abnormalities, high palate with hypertrophic gums, oculocutaneous telangiectasia, and facial freckles (Fig. 1A,C,D). Mild ligament hyperlaxity of upper limbs, clinodactyly of the 5th finger on both hands, asymmetry of the lower limbs, and dorsal scoliosis were also noted.

Since the age of 3 months, the patient had recurrent and frequent episodes of wheezing. The ear-nose-throat evaluation revealed bilateral transmission hearing loss. Ophthalmologic examination was normal and negative for retinoblastoma signs.

Routine metabolic assays, as serum amino acids, urine organic acids, acylcarnitine, and serum alfa-fetoprotein levels were normal. Fragile X CGG repeated test was normal. Peripheral blood white cells were initially radiosensitive to bleomycin, while thereafter, at 11 years of age, they were found resistant to clonogenic survival assay (CSA). Ataxia-telangiectasia mutated (ATM) and meiotic recombination 11(Mre11) protein levels were normal and no point mutations in the *ATM* gene were found.

During the long neurological follow up a progressive improvement of gross and fine motor performances was noted. Tendon reflexes were normal. After 12 years of age, cerebellar ataxia disappeared, but a mild dysmetria and adiadochokinesia persisted. The developmental delay was characterized by impairment of cognitive and speech skills, motor stereotypies, and obsessive behavior. A mild to severe mental retardation was confirmed by Wechsler Intelligence Scale for Children (WISH-R) (IQ 40). At the age of 17, a painful swelling of the left foot, persisting after treatment with steroids and anti-inflammatory, was observed. An X-ray of the foot revealed the presence of sequential atraumatic fractures of the III, IV, and V metatarsus (Fig. 1B).

METHODOLOGY AND RESULTS

Cytogenetic and Molecular Genetics

Karyotype was performed by standard GTG banding at 550 bands resolution (ISCN 2009). Array-CGH was performed with a 44 K whole-genome oligonucleotide microarray Agilent Technologies (Santa Clara, CA) following the manufacturer protocol. Array data were visualized using the Genome Workbench standard edition ver. 5.0 Agilent Technologies (Santa Clara, CA) and compared with the human genome reference sequence hg19 (Feb. 2009).

Real-time quantitive PCR was performed to confirm array-CGH data. We designed a set of primers and probe specific for the exon 8 of gene *ARNTL2* (ref seq NM_020183.3), provided by the Roche Diagnostics Universal probe Library software (http://www.universalprobelibrary.com). Amplification was performed in a total volume of 20 µl containing 2X TaqMan Universal PCR Master mix (P/N 4324018, Applied Biosystems, Foster City, CA), 1X RNaseP kit (20X, VIC dye, P/N 4316844), 0.2 µM of forward and reverse (5′- cgatgccctatgtgacaatg and 5′-ccccctctgcttctaagtaattc) and 0.1 µM UPL probe #46. For each sample a triplicate mix was prepared and aliquoted in three different tubes. The thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles 15 sec 95°C and 1 min 60°C. The PCR was performed on a 7500 fast apparatus (Applied Biosystems, Foster City, CA). The number of gene copies was determined using the comparative delta Ct method.

G-banded chromosomal analysis on peripheral blood lymphocytes indicates normal karyotype. Whole genome array-CGH revealed an interstitial deletion at 13q12.3–q14.11 with breakpoints at 31,955,213 and 43,918,723 bp (Fig. 2) and a 43–167 kb deletion at 12p11.3–p11.22 between 27,182,294 and 27,826,732bp. About 50 genes are located in the 13q deleted region (National Center for Biotechnology Information, hg19, Fig. 2D) and 12 genes in the 12p11.3. The 12p deletion was confirmed by real time PCR centered on exon 8 of the *ARNTL2* gene and showed to be inherited from the asymptomatic father.

Immunological Evaluation

The immunological evaluation revealed increased IgM serum levels (between 240–528 mg/dl, normal range 60–234), with a progressive decrease in the first 7 years of life to stabilize at ~300 mg/dl, well

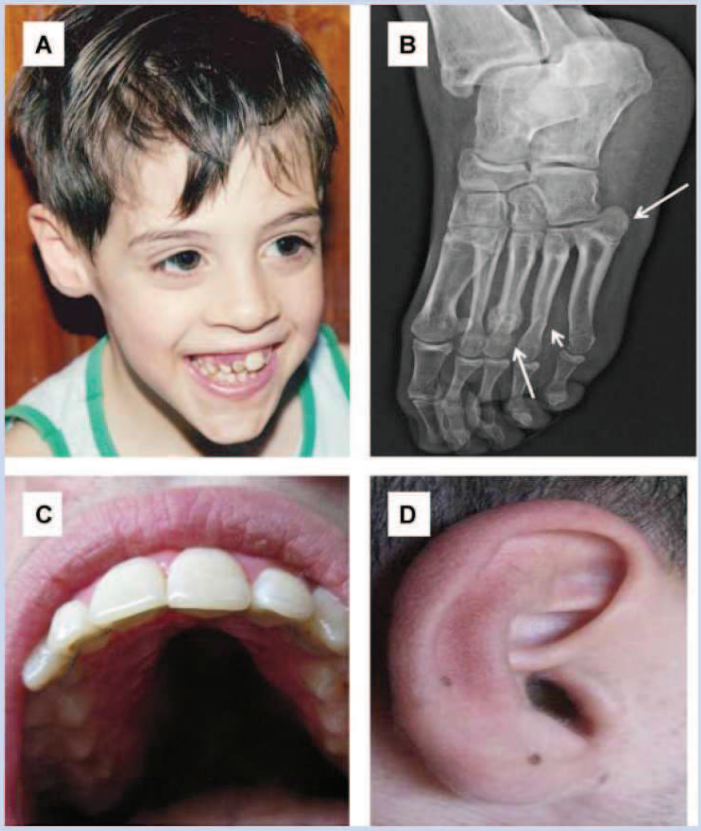

FIG. 1. Proband phenotypic features. A: Noted long face, hypotelorism, teeth abnormalities, and hypertrophic gums; 4 years of age. B: Conspicuous callus repair of the distal diaphysis of the third metatarsal; advanced consolidation of previous fracture of the base of the fifth metatarsal; recent diaphyseal fractures of the distal third of the fourth metatarsus with no reparative callus. C: High-arched palate at 17 years of age. D: Telangiectasias and ear abnormalities.

above the normal upper limit (Fig. 3). IgG and IgA levels were always normal. The patient had normal counts of total white blood cells and lymphocytes. The immunophenotype, evaluated by flow cytometry, revealed normal CD3+, CD4+, CD8+, CD56+, CD3 + HLA-DR+ cells even though the number of CD3 + CD4- CD8- (DN) increased over the time up to 15%. CD19+ cells were in normal range and no significant alterations were observed during the follow up. Normal percentages of memory (CD19 + CD27 + IgM+) and switched memory (CD19 + CD27 + IgM−) were found, representing 14 and 33% of CD19+ cells, respectively. Similarly, naïve (CD19 + IgD + IgM+) and transitional (CD19 + CD27 − IgD+) B cells were within the normal range, representing the 50 and 38%, respectively. As expected, mature B cells (CD19 + CD20 − IgG+) were absent into the peripheral blood (Fig. 4). To exclude that the immunological derangement was due to the chromosome 12 alteration, Ig levels and B cell number were measured in the father. Both determinations were normal (IgG 9.13 g/dl, IgA 2.32 g/dl, IgM 1.56 g/dl; CD19 11%, CD19 + IgD + IgM+ 6.2%, CD19 + CD27 + IgM+ 3.7%, CD19 + CD27 + IgM− 3.1%, CD19 + CD27 − IgD+ 3.7%). Autoantibodies to anti-thyroglobulin (TGB-Ab), anti-thyroid peroxidase (TPO-Ab), anti-nuclear (ANA), anti-double stranded DNA (dsDNA), and anti-transglutaminase were all negative. The proliferative response to mitogens, PHA, and CD3 cross-linking (CD3 XL), was evaluated by thymidine uptake from cultured cells pulsed with 0.5 μCi [^{3}H]thymidine (Amersham International) 8 hr before harvesting. The response to CD3 XL was abnormal in the first 9 years of life, corresponding to 1.5–11% of the control (Fig. 3B). The response to PHA was initially low, corresponding to 23–53% of the control, but variable over the time and became normal by 5 years

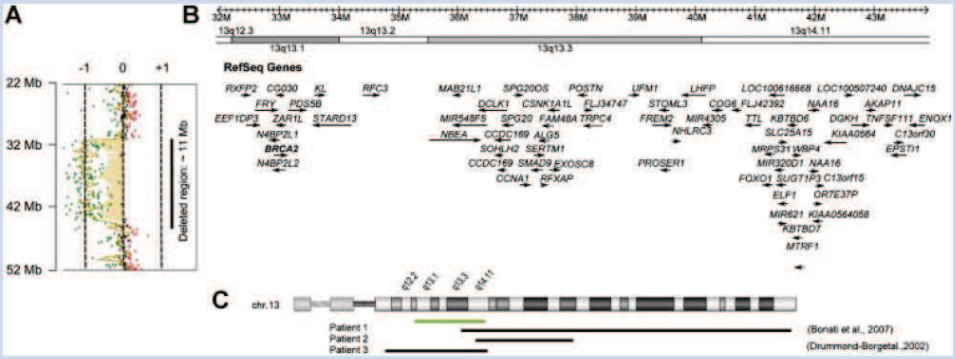

FIG. 2. Array-CGH analysis. A: Array-CGH on chromosome 13. On the y-axis the log ratio is reported [log_2 intensity of (Cy5 fluorocrome/Cy3 fluorocrome)]. Expected values are from −1 to −0.7 for a deletion, 0 for normal, and 0.5 to 1 for a duplication. Distances from the centromere 13q are reported on the left scale. B: The genes involved in the 13q12.3–q14.11 deletion as shown by the UCSC genome browser [http://genome.ucsc.edu/, release hg19]. C: The ideogram of chromosome 13. Green bar indicates the deleted region in our patient and the black bars three partially overlapping patients reported in the literature.

of age. The responses persisted normal thereafter. To better define the pathogenesis of the elevated IgM levels, the in vitro evaluation of class switch recombination (CSR) was studied. CD40 triggering induced a normal B cell proliferation. Moreover, B cells exerted the capability to make the in vitro isotype switch. Bence-Jones proteinuria revealed a prevalence of kappa light chain with a kappa/lambda ratio of 10. In keeping with this finding, cell surface IgM kappa/lambda evaluation revealed a 2.8 ratio. However, no increase of kappa or lambda light chains was found in the serum.

DISCUSSION

We report on a 17-year-old boy carrier of a 12 Mb deletion on 13q with a complex clinical phenotype, characterized by a transient cerebellar ataxia with a mild cerebellar atrophy, elevated IgM levels with transient immunodeficiency. Psychomotor and speech delay, cutaneous telangiectasia and freckles, dysmorphic features, skeletal abnormalities, and spontaneous fractures were also noted. The patient also carried a 12p11.3 deletion unlikely to be of clinical relevance because inherited from the asymptomatic father. In fact, normal serum Ig levels and B cell number were documented in the father.

Three patients partially overlapping the deletion here described are reported (Fig. 2C) [Drummond-Borg et al., 2002; Ballarati et al., 2007; Quélin et al., 2009]. Patient 1 shared with our case mental retardation and cerebellar hypoplasia. Skeletal abnormalities were present in both patient 1 and 2. None reported data on immune response and radiosensitivity.

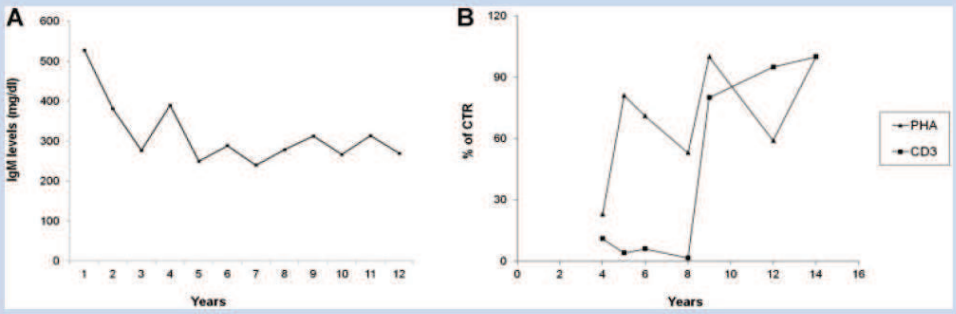

FIG. 3. Immunological evaluation during the long term follow-up. A: A progressive decrease of IgM levels was noted in the first 7 years of life. Thereafter IgM levels remained persistently high. B: An abnormal response to PHA and CD3 XL was noted in the first years of life. The proliferative response to these mitogens became normal by 5 and 9 years of age, respectively.

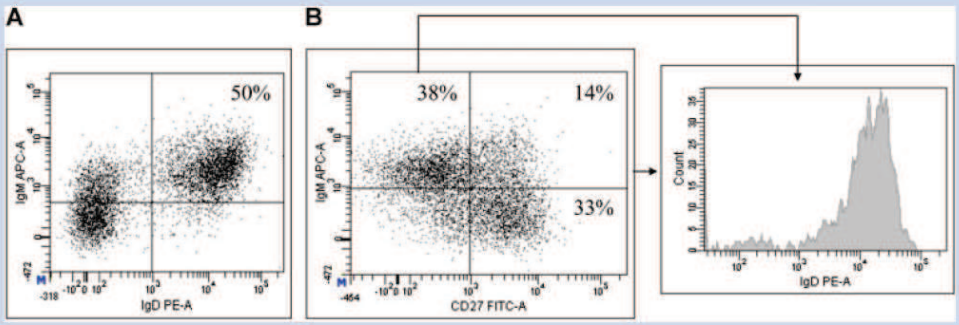

FIG. 4. Blood B cell subsets by flow citometry. A: Naïve B cells (IgD + IgM+) represent the 50% of CD19 + cells. B: Transitional B cells (CD27-IgD +), memory (CD27 + IgM+), and switched memory (CD27 + IgM−) percentages within CD19+ cell population are indicated. Note that CD27-IgM+ cells are also IgD+.

In the patient here described, elevated IgM levels were the immunological hallmark of the syndrome, initially associated with reduced proliferative response to mitogens. The genetics of hyper IgM comprise a heterogeneous group of antibody disorders consisting of five sub-types, characterized by impaired CSR, due to different molecular abnormalities [Kracker et al., 2010]. It should be noted that in our case the initial evaluation of CSR revealed a normal IgG production thus indicating the overall integrity of the CSR B cell machinery. The pathogenesis of some cases is still unknown and, indeed, under certain circumstances it may involve the DNA repair mechanisms. It has been reported that fibroblasts from patients affected with a still molecularly undefined hyper IgM show an increased radiosensitivity, even though less marked than that of patients suffering of other DNA repair defects, such as A-T or Artemis deficiency [Péron et al., 2007]. Moreover, B cell lymphoma and leukemia have been reported in these patients, strongly suggesting a DNA repair defects. It is interesting to note that in our case blood cells were initially found radiosensitive and later during the follow up they were normal to CSA. So far, in none of the reported cases of hyper IgM syndrome the chromosomal aberration herein described has been reported.

As for the radiosensitivity initially observed in the patient here described and not found later during the follow-up, no conclusive explanation is available. Of note an increased cytotoxic effect of X-irradiation has already been documented in patients carrying alterations of chromosome 13, as deletions, trisomy, inversion, or translocation [Nove et al., 1981], being referred to the Rb locus and never in the region of the deletion here described. Thus far, several genes have been associated to altered cellular radiosensitivity, as *ATM*, *p53*, *BRCA1*, *BRCA2*, *DNA-PK* [McKinnon and Caldecott, 2007]. Since *BRCA1* and *BRCA2* genes are essential in preserving the integrity of the genome and it is not clear whether alterations of these genes in heterozygosity may affect the functionality, one possibility is that under certain circumstances, as childhood rapid growth, the haploinsufficiency for these genes may unmask an increased radiosensitivity. Our patient, initially classified as A-T, had some features reminiscent of diseases due to DNA repair defects, and, on the other hand A-T patients may show increased levels of IgM. This finding presumably indicates a chronic antigenic stimulation, since in these patients CSR regularly occurs, as revealed by the ability to produce IgG class antibodies. BRCA1 and BRCA2 proteins are also involved in the repair of DSB by homologous recombination (HR) and their alteration confers high risk for breast and ovarian cancer [Barwell et al., 2007]. Of note, *BRCA2* is also intimately related to Fanconi Anemia (FA), in that *BRCA2* biallelic mutations have recently been identified in group D1 FA (FANCD1) patients. BRCA2 interacts with FA proteins and belongs to a complex functional network, which includes ATM, Rad3 related (ATR) proteins, and *BRCA1* [Wang, 2007] all implicated in DSB.

In conclusion, we describe for the first time a novel clinical phenotype due to a de novo 13q deletion, characterized by elevated IgM levels, transient cerebellar ataxia, mimicking some features of a milder A-T clinical phenotype, which is part of a more complex disease, consisting of mental retardation, dysmorphic features, skeletal abnormalities, and spontaneous bone fractures. As supported by several reports including a fetus showing a de novo mosaic deletion of 13q13.3 [Widschwendter et al., 2002], showing cerebellar hypoplasia and microcephaly associated with multiple abnormalities, we localize the critical chromosomal segment responsible for cerebellar development at 13q13.3.

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REFERENCES

Allderdice PW, Davis JG, Miller OJ, Klinger HP, Warburton D, Miller DA, Allen FHJ, Abrams CA, McGilvray E. 1969. The 13q-deletion syndrome. Am J Hum Genet 21:499–512.

Ballarati L, Rossi E, Bonati MT, Gimelli S, Maraschio P, Finelli P, Giglio S, Lapi E, Bedeschi MF, Guerneri S, Arrigo G, Patricelli MG, Mattina T, Guzzardi O, Pecile V, Police A, Scarano G, Larizza L, Zuffardi O, Giardino D. 2007. 13q Deletion and central nervous system anomalies: Further insights from karyotype-phenotype analyses of 14 patients. J Med Genet 44:e60.

Barwell J, Pangon L, Georgiou A, Kesterton I, Langman C, Arden-Jones A, Bancroft E, Salmon A, Locke I, Kote-Jarai Z, Morris JR, Solomon E, Berg J, Docherty Z, Camplejohn R, Eeles R, Hodgson SV. 2007. Lymphocyte radiosensitivity in BRCA1 and BRCA2 mutation carriers and implications for breast cancer susceptibility. Int J Cancer 121:1631–1636.

Brown LY, Odent S, David V, Blayau M, Dubourg C, Apacik C, Delgado MA, Hall BD, Reynolds JF, Sommer A, Wieczorek D, Brown SA, Muenke M. 2001. Holoprosencephaly due to mutations in ZIC2: Alanine tract expansion mutations may be caused by parental somatic recombination. Hum Mol Genet 10:791–796.

Brown S, Russo J, Chitayat D, Warburton D. 1995. The 13q- syndrome: The molecular definition of a critical deletion region in band 13q32. Am J Hum Genet 57:859–866.

Drummond-Borg M, Kulharya AS, Tonk V, Garcia-Heras J. 2002. Maternal complex chromosome rearrangement ascertained through a del (13) (q12.1q14.1) detected in her mildly affected daughter. Am J Med Genet 107:61–63.

Grace E, Drennan J, Colver D, Gordon RR. 1971. The 13q- deletion syndrome. J Med Genet 8:351–357.

Kivelä T, Tuppurainen K, Riikonen P, Vapalahti M. 2003. Retinoblastoma associated with chromosomal 13q14 deletion mosaicism. Ophthalmology 110:1983–1988.

Kracker S, Gardes P, Mazerolles F, Durandy A. 2010. Immunoglobulin class switch recombination deficiencies. Clin Immunol 135:193–203.

McKinnon PJ, Caldecott KW. 2007. DNA strand break repair and human genetic disease. Annu Rev Genomics Hum Genet 8:37–55.

Nove J, Nichols WW, Weichselbaum RR, Little JB. 1981. Abnormalities of human chromosome 13 and in vitro radiosensitivity; a study of 19 fibroblast strains. Mutat Res 84:157–167.

Péron S, Pan-Hammarström Q, Imai K, Du L, Taubenheim N, Sanal O, Marodi L, Bergelin-Besançon A, Benkerrou M, de Villartay JP, Fischer A, Revy P, Durandy A. 2007. A primary immunodeficiency characterized by defective immunoglobulin class switch recombination and impaired DNA repair. J Exp Med 204:1207–1216.

Quélin C, Bendavid C, Dubourg C, de la Rochebrochard C, Lucas J, Henry C, Jaillard S, Loget P, Loeuillet L, Lacombe D, Rival JM, David V, Odent S, Pasquier L. 2009. Twelve new patients with 13q deletion syndrome: Genotype-phenotype analyses in progress. Eur J Med Genet 52:41–46.

Wang W. 2007. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet 8:735–748.

Widschwendter A, Riha K, Duba HC, Kreczy A, Marth C, SchwS?rzler P. 2002. Prenatal diagnosis of de novo mosaic deletion 13q associated with multiple abnormalities. Ultrasound Obstet Gynecol 19:396–399.

4.4. Conclusive remarks

The description of new clinical phenotype and novel genetic syndrome involving the immune system, opens new scenarios in the molecular mechanisms underlying the immune response.

Our group have documented for the first time a novel pathogenetic mechanism due to an inhibitory anti-lymphocytic autoantibody in a SCID-like phenotype, resulting in total T-cell activation deficiency associated with autoimmunity. Therefore, we reported on severe impairment of T-cell function which was not acquired and induced by viruses.

Of note, we documented an interesting case of children with severe and recurrent infections, even in the absence of a mycobacterial infection, functional and/or genetic alterations of the molecular mechanisms governing Th1/Th2 homeostasis might be responsible for an atypical immunodeficiency and, therefore, should be investigated in these patients.

Eventually, we described a novel clinical phenotype characterized by immunodeficiency with elevated IgM levels, mild and transient cerebellar ataxia, increased frequency of chromosomal breaks, telangiectasia and freckles associated with microcephaly, developmental delay, facial dysmorphisms, skeletal anomalies, and spontaneous fractures, with the chromosomal deletion 13q12.3–q14.11, which includes the BRCA2 gene.

Taken together, these findings demonstrate that important issues on immune system activity and function have to be elucidated.

TECHNOLOGIES

§ Cells and cell cultures

Cord blood (CB) from the FOXN12/2 fetus was obtained by cordocentesis at 16 weeks of gestation. Peripheral Blood Mononuclear cells (PBMC) were obtained from patients and healthy donors by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Leukemia cells were obtained from the bone marrow of newly diagnosed patients, with high leukemia involvement (85-100%), affected by Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), B-precursor Acute Lymphoblastic Leukemia (B-pre ALL) and T-ALL, by using Ficoll-Hypaque (Biochrom) density gradient centrifugation. Normal bone marrow cells were obtained from healthy donors and used as control cells, through the same procedure.

Keratinocytes were isolated by incubation of skin fragments in HBSS ($Ca2^+$ $Mg2^+$ free) containing 0.75% sodium bicarbonate, 100 mM HEPES (Gibco) supplemented with 25 µg/ml Gentamicin with Dispase II (20 mg/ml, Roche). Subsequent removal of epidermal sheet with tweezers, followed by incubation for 10 min at 37°C in 0.05% Trypsin and 0.5 mM EDTA (Gibco). Trypsin-EDTA were stopped adding the same volume of Fetal Bovine Serum (FBS). Cells were filtered through double layer gauze sterilized and then washed with DMEM containing 10% FBS. 5 x 10^6 keratinocytes were plated in 100 mm dish pre-treated with collagen coating solution containing HBSS with 100 µg/ml BSA, 20 mM HEPES pH 6.5, 30 ug/ml bovine type I collagen isolated from dermis (Nutacon). Cells were grown in supplemented Keratinocyte-SFM medium with 30 µg bovine pituitary extract and 0.2 ng/ml human rEGF (Gibco) and 100 IU/ml Penicillin and 100 µg/ml Streptomycin. The cultures were incubated at 37°C in the atmosphere supplemented with 5% CO_2, with the cell culture media changed daily. Fibroblasts were isolated by mincing of dermal skin fragments and were grown in Dulbecco's modified Eagle's medium (Invitrogen) and 10% FBS (Gibco), supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen). The cultures were incubated at 37°C in the atmosphere supplemented with 5% CO_2, with the cell culture media changed daily. Moreover, keratinocytes and fibroblasts were passaged fewer than 5 times before use in experiments.

$CD34^+$ hematopoietic stem cells were isolated by incubating 1-2 x 10^8 PBMCs in 300 µl of PBE with 100µl of CD34 (QBEND 10)-conjugated magnetic beads (Multisort beads, Miltenyi Biotec), followed by incubation for 30 min at 4-8°C. After incubation, the cells were washed with ice-cold PBE (PBS/0.5% bovine serum albumin/5 mM EDTA) and processed through a column placed in a magnetic field and the target cells retained. After washing the column thoroughly with ice-cold PBE, the target cells were recovered by removing the magnetic field and flushing the column with 1 ml of PBE. $CD34^+$ cells were then labeled with a CD34-FITC conjugated antibody for 15-20 min at room temperature.

B lymphoblastoid cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMC using standard procedures.

The human continuous T-acute lymphoblastic leukemia cell line (Molt-4), the chronic myelogenous leukemia cell line (K-562), Burkitt lymphoma cell line and its isogenic derivatives (Raji and Rj225) were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mmol/L L-glutammine (Gibco), and 50 µg/ml gentamycine (Gibco), and cultured at 37°C, 5% CO2. Serum starvation was used to synchronize tumor cells in the G0/G1-phase of the cell cycle. The cells were incubated in medium without FBS for 24 hours. In self-sufficient growth experiments, cells were cultured in DMEM/F12 (Lonza) without FBS and supplemented with 2 mmol/L L-glutammine.

§ Reprogramming of human fibroblasts and iPSCs culture, cleaning and passage

Human wild-type skin fibroblasts (600000 total cells) have been reprogrammed by introducing 3 episomal vector containing cDNA of human *Oct3/4*, *Sox2*, *Klf4* and *L-Myc* (Figure 6) and by using electroporation.

Fibroblasts trasfected have been plated on mitomycin C-treated feeder cells MEFs in a six-well plates, with Fibroblasts Medium Culture without Gentamicin. The next day, the medium has been replaced with new Fibroblasts Medium Culture enriched with Gentamicin. At the 4^{th} day, the medium for primary hES cell culture (hESC medium), supplemented with FGF2 (10 ng/ml), SB431542 (2µM), PD0325901 (0.5 µM), Valproate (500 µM), has been used. hESCs medium has been replaced every 2 days and

the molecule have been added until 14 days after trasfection. Subsequently, the clones were culture

The iPSCs clones have been picked, by cutting around the colonies with a needle mounted on 1mL siringe, until 20 days after trasfection. Of note, the first day of the first two passage was used Rock-Inhibitor to favor clones attachment. To amplify the colonies, they were cut into square pieces of 200µm and they were plated on BJ1-FGF2 feeder layer. The colonies were cleaned from differentiated parts by cutting with a needle and the debris were removed by aspirating the medium culture, which was replace with the new one. After the 5th passage, the clones were cleaned, detached with Dispase (1mg/mL) and freeze by using CryoStor^{TM}CS10 solution.

§ Alkaline phosphatase staining

The culture medium was aspirate from iPS cell culture plates to fix by adding ethanol 95% just to cover the cells. Following, the plates were washed 3 times with PBS 1X. BCIP/NBT substrate solution was added so as to cover the cells, for 10 minutes at 37°C 5% CO_2. The plates were washed 3 times with PBS 1X and dried at RT.

§ Histology

Intestine and liver tissue samples from a 16 weeks FOXN1$^{-/-}$ fetus or control were embedded in OCT compound and snapfrozen in liquid nitrogen or paraffin-embedded. The blocks were cut into serial 5-mm sections and mounted onto microscope slides for H&E staining and immunohistochemistry analysis. Immunodetections were performed by means of a Ventana automat (Ventana Medical Systems).

§ Immunohistochemistry

Tissue sections staining was performed on Benchmark XT platform (Ventana Medical Systems) with pre-diluted CD34, CD20, CD56, CD3, CD4 (Ventana-Confirm), CD8 (Cell Marque), 1:40 CD103 (Beckman Coulter, Brea, CA), 1:50 CD45RA (Dako, Denmark) and 1:25 CD62L Abs (Abcam, Cambridge, UK). Heat antigen retrieval was performed in buffer (CC1, Ventana) following the manufacturer instructions. The slides were incubated with primary Abs at 37u for 32 min (CD34, CD20, CD56, CD3, CD4 and CD8) or for 60 min (CD45RA, CD103, CD62L). Primary Ab was omitted for

negative control. Nuclei were counterstained with hematoxylin. The reaction was detected by the ultraView Universal DAB Detection Kit, which utilizes a cocktail of enzyme labeled secondary Abs that locates the bound primary Ab. The complex is then visualized with hydrogen peroxide substrate and 3, 39-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produces a dark brown precipitate readily detected by light microscopy. Images were acquired by a microscope (DM 2500; Leica, Germany) at magnification 200X or 100X.

§ siRNA transfection

The validated chemically modified oligonucleotides used as siRNA for IL2RG or random non-silencing nucleotides with no known specificity siRNA, used as negative control, were obtained from Invitrogen (Paisley, UK). These siRNAs were transfected at a concentration of 200 pmol/1x10^6 cells in a six well plate for 96 hours. The transfection was performed by the lipid vector Lipofectamine 2000 kit (Invitrogen, Paisley, UK), according to the manufacturer's instructions. Preliminary experiments were performed to establish the silencing efficiency by testing two different oligonucleotides obtained from Invitrogen (Paisley, UK). The amount of protein expression reduction was calculated as follows: 1- (OD_{siRNA} x 100 / $OD_{control\ siRNA}$). In self-sufficient growth experiments, BCLs were cultured in Dulbecco modified Eagle medium (DMEM)/F12 without FBS and supplemented with 2 mM/L L-glutamine.

§ Proliferative assay

Cell proliferation was analyzed by the CFSE dilution assay. Cells (1x10^6) were resuspended in 1 ml PBS-10% FBS and labeled with 1.7 μM CFSE (Molecular Probes). After 2 min in the dark at room temperature, cells were washed in FBS and PBS. After 6 hours cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Cell viability was determined using trypan blue staining. Cell survival was evaluated following stimulation with anti-Fas mAb (400 ng/ml; Upstate) for 6 hours.

Cell proliferation was also analyzed by the thymidine incorporation assay.

For the evaluation in vitro of proliferative response to mitogens of PMBC and CBMC, cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A

(ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories), phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co). 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).

Cells were plated in triplicate at $1x10^5$ viable cells/well in 96-well plates (BD Biosciences), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine for 8 hours before harvesting and the incorporated radioactivity measured by scintillation counting.

In a few experiments complement components were inactivated through heating of serum samples at 56° C for 30 min before use. The percentage of inhibitory activity in the sera was calculated from the formula: (cpm of PHA-stimulated cultures containing 5% tested serum/cpm of PHA-stimulated cultures containing 5% FCS) x 100. Patients and normal IgG were purified using a protein G column according to the vendor's instructions (Pharmacia Biotech).

§ Reagents, western blot and immuneprecipitation

The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences. The Abs anti-γc, anti-JAK3, anti-beta-actin, anti-Bcl-2, anti-Bcl-XL, anti-histone 3 (H3), anti-phosphotyrosine, anti-STAT5, anti-STAT4, anti-BECLIN-1 were purchased from Santa Cruz Biotechnology. The Ab anti-caspase 3 was purchased from Cell Signaling Technology. Acrylamide and bisacrylamide were obtained from Invitrogen. Prestained molecular mass standards were obtained from Bio-Rad. Except where noted, other reagents were from Sigma-Aldrich.

Stimulated or unstimulated cells were washed with ice-cold phosphate buffer saline (PBS; Cambrex, Charles City, IA) and lysed in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na3VO4), 5 μg/ml leupeptin and 5 μg/ml aprotinin on ice for 45 min. The cell lysates were stored at -80°C until processing. Proteins were separated on 12% SDS-PAGE. The membrane was then washed three times in wash buffer and incubated 1 h at room temperature or overnight at 4°C with the specific Ab. The membrane was then washed three times and an appropriate IgG HRP-conjugated secondary Ab was used for the second incubation.

After further washings, the membrane was developed with ECL-developing reagents, and exposed to x-ray films according to the manufacturer's instructions (Amersham Biosciences).

For immunoprecipitation, lysates were normalized for either protein content or cell number and precleared with protein G agarose beads (Amersham Biosciences). The supernatant was incubated with 2 microg/ml anti-JAK3 or polyclonal serum, followed by protein G agarose beads. The immunoprecipitates were separated on density gradient gels, followed by Western blotting. Proteins were detected using antibody for phosphotyrosine.

Densitometric analysis was performed after background equalization through the ImageJ software.

§ Confocal microscopy

After appropriate stimulation, as indicated, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature and centrifuged in a Shandon Cytospin III (Histotronix) onto a glass slide and permeabilized by incubation in a 0.2% Triton X-100 solution for 20 min. BCLs were incubated for 1 h at room temperature with rabbit anti-STAT5 Ab in PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated donkey anti-rabbit IgG (Pierce) in PBS. After washing in PBS, the glass slides were mounted under a coverslip in a 5% glycerol PBS solution. The slides were analyzed by laser scanning confocal microscopy using a Zeiss LSM 510 version 2.8 SP1 Confocal System (Zeiss). At least 100 cells per condition were analyzed in each experiment to determine the rate of STAT5 nuclear translocation.

Intestine and liver tissue samples were blocked with normal goat serum before staining and then treated with 1:50 of PerCP-labeled CD3 (BD Pharmingen, San Diego, CA) and 1:100 of FITC-labeled CD45RA Abs (BD Pharmingen, San Diego, CA) or 1:50 of PE-labeled CD4 (Beckman Coulter, Brea, CA) and 1:50 FITC-labeled CD8 (Beckman Coulter, Brea, CA) or 1:50 APC-labeled CD3 (Beckman Coulter, Brea, CA) and FITC-labeled CD7 (Beckman Coulter, Brea, CA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

§ PCR and quantitative real-time PCR analysis

Total RNA from leukemic samples and controls was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The qPCR reactions were performed in duplicate. The amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche Applied Systems, Germany). The primers used are listed in Table 1. Cycling conditions comprised an initial denaturation at 94°C for 5 min, a phase of annealing/extension for each gene, as follow: 50 cycles at 62°C for 5s (IL2RG); 40 cycles at 62°C for 7s (Cyclin D1); 50 cycles at 62°C for 3 s (Cyclin D2); 40 cycles at 62°C for 7 s (Cyclin D3); 40 cycles at 62°C for 7 s (BCL-XL); 45 cycles at 62°C for 5 s (BECLIN-1), 55 cycles at 62°C for 5sm 35 cycles at 62°C for 20 s (IKAROS, TAL1, SPIB, PTCRA, RAG2, CyclinA1 and CyclinB1) and 72°C for 5 min. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The relative levels of gene expression are represented as $-\Delta Ct=(Ct_{gene}-Ct_{reference})$ and the fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (where Ct is cycle threshold), as previously described.

To evaluate *IL-12Rβ2* chain expression, RNA was reverse transcribed into cDNA using ExpandTM Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim). 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-12Rβ2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. 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.

§ Flow cytometry analysis

At defined time-points (0, 1, 2, 3 and 4 w), the cells cultured on each of the PCL construct were extracted from the scaffolds by aspiration of the medium and flushing of the matrices. Cells were exposed to directly-conjugated mouse anti-human monoclonal antibodies (mAbs) to asses hematopoietic stem cells with CD34-APC (BD Biosciences),

CD45-APC-Cy7 (BD Biosciences), and lymphocyte precursors with CD7-PE (Immunological Sciences), CD1a-FITC (DAKO), CD3-PerCP (BD Biosciences), CD4-PE (BD Biosciences), CD8-PECy7 (Beckman Coulter). The cells were incubated with directly-labelled antibody clones at 4°C in the dark for 30 min, washed and resuspended in 100 ml PBS. The events in the displayed graphs (Figure 4, 5B, 5C) and dot plots (5A, 5D, 5E, 5F, 5G) were gated by forward and side scatter to exclude dead cells. For analysis of early thymocyte subsets with CD7, CD3, CD4 and CD8 T-cell precursors were identified by gating on viable CD45+ cells. Analytical flow cytometry was performed using a BD FACS Canto II flow cytometer (BD Biosciences). Subsequent data processing and preparation for presentation was done using BD FACSDiva software.

In flow cytometry of CBMCs, FITC-, phycoerythrin (PE)-, allophyocyanincyanine 7 (APC-Cy7)-, peridin chlorophyll protein (PerCP)- or PECy7-coupled Abs were used on CB toward CD45 (2D1), CD7 (MT701), CD2 (RPA-2.10), CD3 (UCHT1), CD8a (SK-1), CD8b (2ST8.5H7), CD4 (L200), CD62L (SK11), CD45RA (HI100), CD27 (L128), CD45RO (UCHL-1), CD103 (Ber-ACT8) from BD Pharmingen, San Diego, CA or Beckman Coulter, Brea, CA. FACSCanto II flow cytometer and FACSDiva software (BD Bioscience, San Jose, CA) were used. For each sample, negative controls were stained with irrelevant Abs conjugated with the same fluorochrome. The ‘‘fluorescence-minus-one’’ (FMO) controls have also been used to define precisely the cells that have fluorescence above background levels. Briefly, the samples have been stained with all of the reagents except one.

§ Preparation and characterization of porous scaffolds

3D porous scaffolds were developed by adapting the phase inversion and salt leaching technique. Scaffold morphology was preliminary investigated via FESEM Microscopy (Quanta FEG200, FEI). Specimens were fractured using a razor blade along preferential directions, parallel and perpendicular to the surface. Transverse and longitudinal sections were covered by a thin chromium layer (ca. 20 nm) by automatic sputtering (Emitech K575X) to afford a more efficient electron conductivity of the scaffold surface. The porosity was assessed in terms of pore size, shape and spatial distribution by images at different magnifications and fixed working distance (WD=10 mm). To

obtain a quantitative estimation of the scaffold porosity, three different methods were used: weight measurements by gravimetric method (GM), 2D image analysis (2D-IA) and mercury intrusion porosimetry (MIP). The porosity was obtained by theoretical conditions. The 2D-IA evaluation of porosity features (porosity degree, pore size and spatial distribution) was performed by a dedicated software (ImageJ 1.38b; NIH Freeware; National Institutes of Health, Bethesda). The porosity degree was evaluated from the total surface area of counted pores whereas the pore sizes were derived. Means and standard deviations of pore fraction and size were determined on 10 different SEM images. Porosity measurements by MIP were assessed to estimate the really interconnected pores and their specific pore surface. A mercury surface tension of 480 mN/m and a contact angle of 141.38° were used, while a pressure gradient from 400 Pa up to 200 KPa was intruded to exactly count either micro and macropores according to the Washburn equation.

§ Scanning Electron Microscopy (SEM)

As for the investigation of biohybrid scaffold, cells were fixed for 2 h in 2,5% glutaraldehyde solution and dehydrated with sequential washes in 50%, 70%, 80%, 90% and 100% ethanol. The samples were air-dried overnight before the chromium sputtering. In this case, the accelerating voltage of the FESEM equipment (Quanta FEG200, FEI) was set at 5 kV, reducing the vacuum level into the chamber (LV or Low Vacuum mode), so preventing any negative interaction of electron beam with the cellular bodies.

§ Cytogenetic analysis

Karyotype was performed by standard GTG banding at 550 bands resolution (ISCN 2009). Array-CGH was performed with a 44 K whole-genome oligonucleotide microarray Agilent Technologies (Santa Clara, CA) following the manufacturer protocol. Array data were visualized using the Genome Workbench standard edition ver. 5.0 Agilent Technologies (Santa Clara, CA) and compared with the human genome reference sequence hg19 (Feb. 2009).

§ Statistical analysis

Microsoft Excel and *MedCalc* were used for data analysis. The correlations between gene expression was obtained using the Pearson's correlation. All measurements were expressed as the mean $\pm$ SE. A value of $p < 0.05$ values was considered statistically significant.

REFERENCES

1. Delves PJ, Roitt IM. The Immune System. First of two parts. *N Engl J Med* 343:37-49, 2000.

2. Delves PJ, Roitt IM. The Immune System. Second of two parts. *N Engl J Med* 343:108-117, 2000.

3. Parkin J, Cohen B. An overview of the immune system. *Lancet* 375:1777-89, 2001.

4. Notarangelo LD, Fischer A, Geha RS, et al. Primary immunodeficiencies: 2009 update. *J Allergy Clin Immunol* 124:1161-78, 2009.

5. Sponzilli I, Notarangelo LD. Severe combined immunodeficiency (SCID): from molecular basis to clinical management. *Acta Biomed*. 82:5-13, 2011.

6. Amman AJ, Wara DW, Pillarisetty RJ, et al. The prevalence of autoantibodies in T-cell, and phagocytic immunodeficiency disorders. *Clin Immunol Immunopathol* 14:456-66, 1979.

7. Carneiro-Sampaio M, Coutinho A. Tolerance and autoimmunity: lessons at the bedside of primary immunodeficiencies. *Adv Immunol* 95:51-82, 2007.

8. Notarangelo LD, Gambineri E, Badolato R. Immunodeficiencies with autoimmune consequences. *Adv Immunol* 89:321-70, 2006.

9. Fairweather D, Kaya Z, Shellan GR, et al. From infection to autoimmunity. *J Autoimmune* 16:341-45, 2001.

10. Fischer A, Landais P, Friedrich W, et al. European experience of bone-marrow transplantation for severe combined immunodeficiency. *Lancet* 336:850-4, 1990.

11. Buckley RH. Primary cellular immunodeficiencies. *J Allergy Clin Immunol* 109:747-57, 2002.

12. Buckley RH. Advances in the understanding and treatment of human severe combined immunodeficiency. *Immunol Res* 22:237-51, 2000.

13. Nehls, M., Pfeifer D, Schorpp M, et al. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372:103, 1994.

14. 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* 65:167, 1996.

15. Frank J, Pignata C, Panteleyev AA, et al. Exposing the human nude phenotype. Nature 398:473, 1999.

16. Duijvestijn AM, Hoefsmit EC. Ultrastructure of the rat thymus: the microenvironment of T-lymphocyte maturation. *Cell TissueRes* 218:279-92, 1981.

17. van Ewijk W. T-cell differentiation is influenced by thymic microenvironments. *Ann Rev Immunol* 9:591–615, 1991.

18. Dorshkind K. Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol* (1990) 8:111–37, 1990.

19. Manley NR, BlackburnCC. A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? *Curr Opin Immunol* 15:225-32, 2003.

20. Manley NR. Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin Immunol* 12:421, 2000.

21. Palmer DB, Viney JL, Ritter MA, et al. Expression of the alpha beta T-cell receptor is necessary for the generation of the thymic medulla. *Dev Immunol* 3:175-9, 1993.

22. Owen JJ, Ritter MA. Tissue interaction in the development of thymus lymphocytes. *J ExpMed* 129:431-42, 1969.

23. Calderon L, BoehmT. Three chemokine receptors cooperatively regulate homing of hematopoietic progenitors to the embryonic mouse thymus. *Proc Natl Acad Sci U SA* 108:7517-22, 2011.

24. Liu C, Saito F, LiuC, et al. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* 108:2531-9, 2006.

25. Wurbel MA, Malissen M, Guy-Grand D, et al. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor gamma delta(C) gut intraepithelial lymphocytes. *Blood* 98:2626-32, 2001.

26. Thompson PK, Zúñiga-Pflücker JC. On becoming a T cell, a convergence of factors kick it up a Notch along the way. *Semin Immunol* 23:350-9, 2011.

27. Hozumi K, Mailhos C, Negishi N, et al.Delta-like4 is indispensable in thymic environment specific for T cell development. *J ExpMed* 205:2507-13, 2008.

28. Lind EF, Prockop SE, Porritt HE, et al. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J ExpMed* 194:127-34, 2001.

29. Benz C, Heinzel K, Bleul CC. Homing of immature thymocytes to the subcapsular microenvironment within the thymus is not an absolute requirement for T cell development. *Eur J Immunol* 34:3652-63, 2004.

30. Lee CK, Kim JK, Kim Y, et al. Generation of macrophages from early T progenitors in vitro. *J Immunol* 166: 5964-69, 2001.

31. Schmitt TM, Ciofani M, Petrie HT, et al. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* 200:469-79, 2004.

32. Talebian L, Li Z, Guo Y, et al. T-lymphoid, megakaryocyte, and granulocyte development are sensitive to decreases in CBFβ dosage. *Blood* 109:11-21, 2007.

33. Inoue J, Kanefuji T, Okazuka K, et al. Expression of TCRαβ partly rescues developmental arrest and apoptosis of αβ T cells in Bcl11b$^{-/-}$ mice. *J Immunol* 176:5871-79, 2006.

34. Taghon TN, David ES, Zũniga-Pflücker JC, et al. Delayed, asynchronous, and reversible T lineage specification induced by Notch/Delta signaling. *Genes and Development* 19:965-78, 2005.

35. Shimizu C, Kawamoto H, Yamashita M, et al. Progression of T cell lineage restriction in the earliest subpopulation of murine adult thymus visualized by the expression of lck proximal promoter activity. *Int Immunol* 13:105-17, 2001.

36. Anderson G, Jenkinson WE, Jones T, et al. Establishment and functioning of intrathymic microenvironments. *Immunol Rev* 209:10-27, 2006.

37. von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. *Annu Rev Immunol* 15:433-52, 1997.

38. Fehling HJ, von Boehmer H. Early αβ T cell development in the thymus of normal and genetically altered mice. *Curr Opin Immunol* 9:263-75, 1997.

39. Kisielow P, Teh HS, Bluthmann H, et al. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 335:730-33, 1988.

40. Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Annu Rev Immunol* 13:93-126, 1995.

41. Bousso P, Bhakta NR, Lewis RS, et al. Dynamics of thymocyte-stromal cell interactions visualized by twophoton microscopy. *Science* 296:1876-80, 2002.

42. Bendelac A, Matzinger P, Seder RA, et al. Activation events during thymic selection. J Exp Med 175:731-42, 1992.

43. Ramsdell F, Jenkins M, Dinh Q, et al. The majority of CD4+8− thymocytes are functionally immature. *J Immunol* 147:1779-85, 1991.

44. van Ewijk W, Hollander G, Terhorst C, et al. Stepwise development of thymic microenvironments in vivo is regulated by thymocyte subsets. *Development* 127:1583-91, 2000.

45. Klug DB, Carter C, Gimenez-Conti IB, et al. Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. J Immunol 169:2842-45, 2002.

46. Boehm, T, Bleul CC, Schorpp M. Genetic dissection of thymus development in mouse and zebrafish. *Immunol Rev* 195:15, 2003.

47. Owen JJ, Ritter MA. Tissue interaction in the development of thymus lymphocytes. *J Exp Med* 129:431-63, 1969.

48. Haynes BF, Heinley CS. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into fetal thymic microenviroment. *J Exp Med* 181:1445-58, 1995.

49. Schorpp M, Hoffmann M, Dear TN, et al. Characterization of mouse and human nude genes. *Immunogenetics* 46:509, 1997.

50. Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 8:295, 1966.

51. Nehls MB, Kyewski M, Messerle R, et al. Two genetically separable steps in the differentiation of thymic epithelium. *Science* 272:886, 1996.

52. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* 96:857-68, 1999.

53. Kops GJPL, de Ruiter ND, De Vries-Smits AMM, et al. Direct control of the forkhead transcription factor AFX by protein kinase B. *Nature* 398:630-34, 1999.

54. Balciunaite G, Keller MP, Balciunaite E, et al. Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice. *Nat immunol* 3:1102-08, 2002.

55. Tsai PT, Lee RA, Wu H. BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood* 102:3947-53, 2003.

56. Jonsson H, Peng SL. Forkhead transcription factors in immunology. *CMLS Cell Mol Life Sci* 62:397-409, 2005.

57. Ye H, Kelly TF, Samadani U, et al. Hepatocyte nuclear factor 3/fork head homolog 11 is expressedin proliferating epithelial and mesenchymal cells of embryonic and adult tissues. *Mol Cell Biol* 17:1626-41, 1997.

58. Takahashi Y, Shimizu A, Sakai T, et al. Mapping of the nu gene using congenic nude strains and in situ hybridization. *J Exp Med* 175:873-76, 1992.

59. Byrd LG. Regional localization of the nu mutation on chromosome 11. *Immunogenetics* 37:157-9, 1993.

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

61. Shakib S, Desanti GE, Jenkinson WE, et al. Checkpoints in the development of thymic cortical epithelial cells. *J Immunol* 182:130-7, 2009.

62. Manley NR, Condie BG. Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. *Prog Mol Biol Transl Sci* 92:103-20, 2010.

63. Jerome LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene,Tbx1. *Nat Genet* 27:286-91, 2001.

64. Dietrich S,Gruss P. Undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. *Dev Biol* 167:529-48, 1995.

65. Hetzer-Egger C, Schorpp M, Haas-Assenbaum et al. Thymopoiesis requires Pax9 function in thymic epithelial cells. *Eur J Immunol* 32:1175-81, 2002.

66. Su DM, Manley NR. Hoxa3 and pax1 transcription factors regulate the ability of fetal thymic epithelial cells to promote thymocyte development. *J Immunol* 164:5753-60, 2000.

67. Manley NR, Capecchi MR. The role of Hoxa-3 in mouse thymus and thyroid development. *Development* 121:1989-2003, 1995.

68. Zou D, Silvius D, Davenport J, et al. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Dev Biol* 293:499-512, 2006.

69. Rodewald HR. Thymus organogenesis. *Annu Rev Immunol* 26:355-88, 2008.

70. Rossi SW, Jenkinson WE, Anderson G, et al. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* 441:988-91, 2006.

71. Chen L, Xiao S , Manley NR . Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. *Blood.* 113:567-74, 2009.

72. Corbeaux T, Hess I, Swann JB, et al. Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage. *Proc Natl Acad Sci USA* 107:16613-8, 2010.

73. Crum CP, McKeon FD. p63in epithelial survival, germ cell surveillance, and neoplasia. *Annu Rev Pathol* 5:349-71, 2010.

74. Candi E, Rufini A, Terrinoni A, et al. DeltaNp63 regulates thymic development through enhanced expression of FgfR2 and Jag2. *Proc Natl Acad Sci U SA* 104:11999-2004, 2007.

75. Lee D, Prowse DM, Brissette JL. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. *Dev Biol* 208:362-74, 1999.

76. Hardy MH. The secret life of the hair follicle. *Trends Genet* 8:55-61, 1992.

77. Clark RA, Yamanaka K, Bai M, et al. Human skin cells support thymus-indipendent T-cell development. *J Clin Invest* 115:3239-49, 2005.

78. Pantelouris EM, Hair J. Thymus dysgenesis in nude (nu/nu) mice. *J Embryol Exp Morphol* 24:615-23, 1970.

79. Hofmann M, Harris M, Juriloff D, et al. Spontaneous mutations in SELH/Bc mice due to insertions of early transposons: molecular characterization of null alleles at the nude and albino loci. *Genomics* 52:107, 1998.

80. 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* 141:647-48, 2005.

81. Adriani M, Martinez-Mir A, Fusco F, et al. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. *Ann Hum Genet* 68:265-68, 2004.

82. Vigliano I, Gorrese M, Fusco A, et al. FOXN1 mutation abrogates prenatal T-cell development in humans. *J Med Genet* 48:413-16, 2011.

83. Peaudecerf L, Ribeirodos Santos P, Boudil A, et al. The role of the gut as a primary lymphoid organ: CD8 euthymic mice derive from very immature CD44C thymocyte precursors. *Mucosal Immunol* 4:93-101, 2011.

84. Blais M, Louis I, Perreault C. T-cell development: an extrathymic perspective. *Immunol Rev* 209:103-14, 2006.

85. Nonaka S, Naito T, Chen H, et al. Intestinal γδ T cells develop in mice lacking thymus, all lymphonodes, Peyer's patches, and isolated lymphoid follicles. *J Immunol* 174:1906-12, 2005.

86. Xiong N, Raulet DH. Development and selection of gamma delta T cells. *Immunol Rev* 215:15-31, 2007.

87. Hayday A, Gibbons D. Brokering the peace: the origin of intestinal T cells. *Mucosal Immunol* 1:12-4, 2008.

88. Gunther U, Holloway JA, Gordon JG, et al. Phenotypic characterization of CD3-7C cells in developing human intestine and an analysis of their ability to differentiate into T cells. *J Immunol* 174:5414-22, 2005.

89. Haddad R, Gulmiot F, Six E, et al. Dynamics of Thymus-Colonizing Cells during Human Development. *Immunity* 24:217-30, 2006.

90. Sanchez MJ, Gutierrez-Ramos JC, Fernandez E, et al. Putative Prethymic T Cell Precursors within the Early Human Embryonic Liver: A Molecular and Functional Analysis. *J Exp Med* 177:19-33, 1993.

91. Haynes BF, Martin ME, Kay HH, et al. Early events in human T cell ontogeny. Phenotypic characterization and immunohistologic localization of T cell precursors in early human fetal tissues. *J Exp Med* 168:1061-80, 1989.

92. Eiras P, Leon F, Camarero C, et al. Intestinal intraepithelial lymphocytes contain a CD3-CD7+ subset expressing natural killer markers and a singular pattern of adhesion molecules. *Scand J Immunol* 52:1-6, 2000.

93. Rocha B. The extrathymic T-cell differentiation in the murine gut. *Immunol Rev* 215:166-77, 2007.

94. Lin T, Matsuzaki G, Kenai H, et al. Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes. *Eur J Immunol* 23:1968-74, 1993.

95. Oida T, Suzuki K, Nanno M, et al. Role of Gut Cryptopatches in Early Extrathymic Maturation of Intestinal Intraepithelial T Cells. *J Immunol* 164:3616-26, 2000.

96. Guy-Grand D, Azogui O, Celli S, et al. Extrathymic T Cell Lymphopoiesis: Ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. *J Exp Med* 197:333-41, 2003.

97. Kuo TC, Schlissel MS. Mechanisms controlling expression of the RAG locus during lymphocyte development. *Curr Opin Immunol* 21:173-8, 2009.

98. Bruno L, Rocha B, von Boehmer H, Rodewald H-R. Intra- and extra-thymic expression of the pre-T cell receptor α gene. *Eur J Immunol* 25:1877-82, 1995.

99. von Boehmer H, Fehling HJ. Structure and function of the pre-T cell receptor. *Annu Rev Immunol* 15:433-52, 1997.

100. David-Fung ES, Butler R, Buzi G, et al. Transcription factor expression dynamics of early T-lymphocyte specification and commitment. *Dev Biol* 325:444-67, 2009.

101. Bas A, Hammarstrom SG, Hammarstrom MLKC. Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. *J Immunol* 171:3359-71, 2003.

102. Hillion S, Rochas C, Youinou P, Jamin C. Signaling pathways regulating RAG expression in B lymphocytes. *Autoimmunity Rev* 8:599-604, 2009.

103. Howie D, Spencer J, DeLord D, et al. Extrathymic T cell differentiation in the human intestine early in life. *J Immunol* 161:5862-72, 1998.

104. Uss E, Rowshani AT, Hooibrink B, et al. CD103 is a marker for alloantigen-induced regulatory CD8+ T cells. *J Immunol* 177:2775-83, 2006.

105. Nowell CS, Bredenkamp N, Tetélin S, et al. Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. *PLoS Gene* 7(11):e1002348, 2011.

106. Guo J, Rahman, M, Cheng, L, et al. Morphogenesis and maintenance of the 3D-thymic medulla and prevention of nude skin phenotype require FoxN1 in pre- and post-natal K14 epithelium. *J Mol Med* 89:263-77, 2011.

107. Coffer PJ, Burgering BM. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* 4:889, 2004.

108. Romano R, Palamaro L, Fusco A, et al. From murine to human nude/SCID: the thymus, T-cell development and the missing link. *Clin Dev Immunol* 2012:467101, 2012.

109. Laster AJ, Itoh T, Palker T J, et al. The human thymic microenvironment: thymic epithelium contains specific keratins associated with early and late stages of epidermal keratinocyte maturation. *Differentiation* 31:67, 1986.

110. Radtke F, Fasnacht, N, Macdonald HR. Notch signaling in the immune system. *Immunity* 32:14, 2010.

111. Ewijk W van, Wang B, Hollander GA, et al. Thymic microenvironments, 3-D versus 2-D? *Semin Immunol* 11:57-64, 1999.

112. Haddad R, Guimiot, F, Six E, et al. Dynamics of thymus-colonizing cells during human development. *Immunity* 24:217, 2006.

113. Jaleco AC, Neves H, Hooijberg E, et al. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med* 194: 991–1002, 2001.

114. Schmitt TM, Zúñiga-Pflücker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17:749-56, 2002.

115. Spits H, Blom, B, Jaleco, AC. et al. Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol Rev* 165:75, 1998.

116. Herblot S, Steff AM, Hugo P, et al. SCL and LMO1 alter thymocyte differentiation: inhibition of E2A-HEB function and pre-T alpha chain expression. *Nat Immunol* 1:138-44, 2000.

117. Ikawa T, Fujimoto S, Kawamoto H, et al. Commitment to natural killer cells requires the helix-loop-helix inhibitor Id2. *Proc Natl Acad Sci USA* 98:5164-69, 2001.

118. Anderson MK. At the crossroads: diverse roles of early thymocyte transcriptional regulators. *Immunol Rev* 209: 191-211, 2006.

119. Zhang Y, Payne KJ, Zhu Y, et al. SCL expression at critical points in human hematopoietic lineage commitment. *Stem Cells* 23:852, 2005.

120. Brunet de la Grange P, Armstrong F, Duval V, et al. Low SCL/TAL1 expression reveals its major role in adult hematopoietic myeloid progenitors and stem cells. *Blood* 108:2998, 2006.

121. Rothenberg EV. Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. *Immunity* 26:690, 2007.

122. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131:861-72, 2007.

123. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663-76, 2006.

124. Okita K, Hong H, Takahashi K, et al. Generation of mouse-induced pluripotent stem cells with plasmid vectors. *Nature Protocol* 5:418-28, 2010.

125. Bellin M, Marchetto MC, Gage FH. Induced pluripotent stem cells: the new patient? *Nature Rev Mol Cell Biol* 13:713-726, 2012.

126. Cavazzana-Calvo M, Carlier F, Le Deist F, et al. Long,term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. *Blood* 109:4575-81, 2007.

127. Taga T, Kishimoto T. Cytokine receptors and signal transduction. *FASEB J* 7:3387-96, 1993.

128. Timmermann A, Pflanz S, Grotzinger J, et al. Different epitopes are required for gp130 activation by interleukin,6, oncostatin M and leukemia inhibitory factor. *FEBS J* 468:120-4, 2000.

129. Leonard WJ. The molecular basis of X-inked severe combined immunodeficiency: defective cytokine receptor signaling. *Annu Rev Med* 47:229-39, 1996.

130. Lindemann MJ, Benczik M, Gaffen SL. Anti-apoptotic signalling by the interleukin-2 receptor reveals a function for cytoplasmic tyrosine residues within the common gamma (gamma c) receptor subunit. *J Biol Chem*;278:10239-49, 2003.

131. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by γc family cytokines. *Nat Rev Immunol* 9:408-490, 2009.

132. Miyazaki T, Kawahara A, Fujii H, et al. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 266:1045-7, 1994.

133. Egan SE, Giddings BW, Brooks MW, et al. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 363:45-51, 1993.

134. Johnston JA, Wang L, Hanson EP, et al. Interleukins 2, 4, 7 and 15 stimulate tyrosine phosprorylation of insulin receptor substrates 1 and 2 in T cells. *J Biol Chem* 270:28527-30, 1995.

135. Ivashkiv LB, Hu X. Signaling by STATs. *Arthritis Res Ther* 6:159-68, 2004.

136. Adriani M, Garbi C, Amodio G, Russo I, Giovannini M, Amorosi S, et al. Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus. J Immunol. 2006;177:6889,95.

137. Fuh G, Mulkerrin MG, Bass S, et al. The human growth hormone receptor. *J Biol Chem* 265:3111-5, 1990.

138. Gaspar HB, Parsley KL, Howe S, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 364:2181-7, 2004.

139. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302:415-9, 2003.

140. Aiuti A, Slavin S, Aker M, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 296:2410-3, 2002.

141. Kohn DB, Sadelain M, Glorioso JC. Occurrence of leukaemia following gene therapy of X,linked SCID. *Nat Rev Cancer* 3:477-88, 2003.

142. Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, Kaiser C, et al. Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J* 18:3064-73, 1999.

143. Conway Campbell BL, Wooh JW, Brooks AJ, et al. Nuclear targeting of the growth hormone receptor results in dysregulation of cell proliferation and tumorigenesis. *PNAS* 104:13331-6, 2007.

144. Swerdlow AJ. Does growth hormone therapy increase the risk of cancer? *Nat Clin Pract Endocrinol Metab* 2:530-1, 2006.

145. Cabanas P, Garcia-Caballero T, Barreiro J, et al. Papillary thyroid carcinoma after recombinant GH therapy for Turner syndrome. *Eur J Endocrinol* 153:499-502, 2005.

146. Ogilvy, Stuart AL, Gleeson H. Cancer risk following growth hormone use in childhood: implications for current practice. *Drug Saf* 27:369-82, 2004.

147. Havens CG, Ho A, Yoshioka N, et al. Regulation of Late G1/S Phase Transition and APCCdh1 by Reactive Oxygen Species. *Mol Cell Biol* 26:4701-11, 2006.

148. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes hematopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440-42, 1988.

149. Strasser A, Harris AW, Cory S. Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67, 889-899, 1991.

150. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 100:57-70, 2000.

151. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108:153-64, 2002.

152. Sherr CJ, Roberts JM. Living with or without cyclins and cyclin,dependent kinases. *Genes Dev* 18:2699-7112004.

153. Lord JD, McIntosh BC, Greenberg PD, et al. The IL-2 Receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *J Immunol* 164:2533-41, 2000.

154. Martino A, Holmes IV JH, Lord JD, et al. Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-21. *J Immunol* 166:1723-9, 2001.

155. Moon JJ, Rubio ED, Martino A, et al. A permissive role for phosphatidylinositol 3-kinase in the Stat5,mediated expression of cyclin D2 by the interleukin-2 receptor. *J Biol Chem* 279:5520-7, 2004.

156. Engedal N, Gjevik T, Blomhoff R, et al. All-trans retinoic acid stimulates IL-2-mediated proliferation of human T lymphocytes: early induction of cyclin D3. *J Immunol* 177:2851-61, 2006.

157. Lamkanfi M, Festjens N, Declercq W, et al. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* 14:44-55, 2007.

158. Tait SWG, Green DR. Caspase independent cell death: leaving the set without the final cut. *Oncogene* 27:6452-61, 2008.

159. Haraguchi BM, Torii S, Matsuzawa S, et al. Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. *J Exp Med* 191:1709-20, 2000.

160. Hua F, Cornejo MG, Cardone MH, et al. Effects of Bcl-2 levels on Fas signaling-induced Caspase-3 activation: Molecular genetic tests of computational model predictions. *J Immunol* 175:985-95, 2005.

161. von Freeden, Jeffry U, Solvason N, et al. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity* 7:147-54, 1997.

162. Akbar AN, Borthwick NJ, Wickremasinghe RG, et al. Interleukin-2 receptor common gamma-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro, apoptotic (bax, bcl-xS) gene expression. *Eur J Immunol* 26:294-9, 1996.

163. Zhou F, Yang Y, Xing D. Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS J* 278:403-13, 2011.

164. Ekberg J, Holm C, Jalili S, Richter J, Anagnostaki L, Landberg G, et al. Expression of cyclin A1 and cell cycle proteins in hematopoietic cells and acute myeloid leukemia and links to patient outcome. *Eur J Haematol* 75:106-15, 2005.

165. Budirahardja Y, Gonczy P. Coupling the cell cycle to development. *Development* 136:2861-72, 2009.

166. Yam CH, Fungand TK, Poon RYC. Cyclin A in cell cycle control and cancer. *Cell Mol Life Sci* 59:1317-26, 2002.

167. Yuan J, Yan R, Kramer A, et al. Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells. *Oncogene* 23:5843-52, 2004.

168. Hochegger H, Takeda S, Hunt T. Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nature* 9:910-6, 2008.

169. Kalaszczynska I, Geng Y, Lino T, et al. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. *Cell* 138:352-65, 2009.

170. Landis MW, Pawlyk BS, Li T, et al. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. *Cancer Cell* 9:13-22, 2006.

171. Decker T, Schneller F, Hipp S, et al. Cell cycle progression of chronic lymphocytic leukemia cells is controlled by cyclin D2, cyclin D3, cyclin-dependent kinase (cdk) 4 and the cdk inhibitor p27. *Leukemia* 16:327-34, 2002.

172. Sicinska E, Aifantis I, Le Cam L, et al. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4:451-61, 2003.

173. Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. *Cell* 118:477-91, 2004.

174. Amorosi S, Russo I, Amodio G, et al. The cellular amount of the common c-chain influences spontaneous or induced cell proliferation. *J Immunol* 182:3304-09, 2009.

175. Vigliano I, Palamaro L, Bianchino G. Role of the common γ chain in cell cycle progression of human malignant cell lines. *Int Immunol* 24:159-167, 2012.

176. Barata JT, Keenan TD, Silva A, et al. Common γ chain-signaling cytokines promote proliferation of T-cell acute lymphoblastic leukemia. *Haematol* 89:1459-67, 2004.

177. Ribeiro D, Melão A, Barata JT. IL-7R-mediated signaling in T-cell acute lymphoblastic leukemia. *Adv Biol Regul* 53:211-22, 2013.

178. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* 13:261-91, 1997.

179. Jayaraman A, Jamil K. Drug targets for cell cycle dysregulators in leukemogenesis: in silico docking studies. 15;9:e86310, 2014.

180. Cao X, Fan L, Fang C. The expression of SOX11, cyclin D1, cyclin D2, and cyclin D3 in B-cell lymphocytic proliferative diseases. *Med Oncol* 29:1190-6, 2012.

181. Cooper GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates, 2000.

182. Dewson G, Kluck RM. Bcl-2 family-regulated apoptosis in health and disease. *Cell Health Cytoskeleton* 2:9-22, 2010.

183. Scarfò L, Ghia P. Reprogramming cell death: BCL2 family inhibition in hematological malignancies. *Immunol Lett* 155:36-39, 2013.

184. Romano R, Palamaro L, Parenti G, et al. Networking Between γc and GH-R Signaling in the Control of Cell Growth. Curr Sign Trans Ther 8:67-73, 2013.

185. Roy S, Debnath J. Autophagy and tumorigenesis. *Semin Immunopathol* 32:383-96, 2010.

186. Wang RC, Levine B. Autophagy in cellular growth control. *FEBS Lett* 584:1417-26, 2010.

187. Rosenfeldt MT, Ryan KM. The role of autophagy in tumour development and cancer therapy. *Expert Rev Mol Med* 11:e36, 2009.

188. Nakase K , Kit K, Miwa H . Clinical and prognostic significance of cytokine receptor expression in adult acute lymphoblastic leukemia: interleukin-2 receptor α-chain predicts a poor prognosis. *Leukemia* 21:326-32, 2007.

189. Zetterberg A, Larsson O, Wiman K. What is the restriction point? *Curr Opin Cell Biol* 7:835-42, 1995.

190. Morgan D. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol* 13:261-91, 1997.

191. Cordon-Cardo C. Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 147:545-60, 1995.

192. Cao X, Fan L, Fang C, et al. The expression of SOX11, cyclin D1, cyclin D2, and cyclin D3 in B-cell lymphocytic proliferative diseases. *Med Oncol* 29:1190-96, 2012.

193. Volm M, Koomägi R, Stammler G, et al. Prognostic implications of cyclins (D1, E, A), cyclin-dependent kinases (CDK2, CDK4) and tumor-suppressor genes (pRB, p16INK4A) in childhood acute lymphoblastic leukemia. *Int J Cancer* 74:508-12, 1997.

194. Aref S, Mabed M, El-Sherbiny M, et al. Cyclin D1 expression in acute leukemia. Hematology 11:31-4, 2006.

195. Sicinska E, Aifantis I, Le Cam L, et al. Requirement for cyclin D3 in lymphocyte development and T cell leukemias. Cancer Cell 4:451-61, 2003.

196. Addeo R, Caraglia M, Baldi A, et al. Prognostic Role of bcl-xL and p53 in Childhood Acute Lymphoblastic Leukemia (ALL). *Canc Biol Ther* 4:32-8, 2005.

197. Racay P, Hatok J, Hudecek J, et al. Transcription of genes of p53-dependent apoptosis in acute leukaemia. *Inter J Mol Med* 22:833-39, 2008.

198. Sato K, Tsuchihara K, Fujii S, et al. Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. *Cancer Res* 67:9677-684, 2007.

199. Moreau K, Luo S, Rubinsztein DC. Cytoprotective roles for autophagy. *Curr Opin Cell Biol* 22: 206-11, 2010.

200. Jin S, White E. Role of autophagy in cancer: management of metabolic stress. *Autophagy* 3: 28-31, 2007.

201. Degenhardt K, Mathew R, Beaudoin B, et al. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 10:51-64, 2006.

202. Cao Y, Klionsky DJ. Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res* 17:839-49, 2007.

203. Liang C, Feng P, Ku B, et al. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* 8:688-99, 2006.

204. Fimia GM, et al. Ambra1 regulates autophagy and development of the nervous system. *Nature* 447:1121-25, 2007.

205. Takahashi Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 9:1142-51, 2007.

206. Mazza C, Buzi F, Ortolani F, et al. Clinical heterogeneity and diagnostic delay of autoimmune polyendocrinopathy-candidiasisectodermaldystrophy syndrome. *Clin Immunol* 139: 6-11, 2011.

207. Capalbo D, Fusco A, Aloj G, et al. High intrafamilial variability in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy: a case study. *J Endocrinol Invest* 35:77-81, 2012.

208. Capalbo D, Mazza C, Giordano R, et al. Molecular background and genotype-phenotype correlation in autoimmune-polyendocrinopathycandidiasis-ectodermal-distrophy patients from Campania and in their relatives. *J Endocrinol Invest* 35:169-73, 2012.

209. A. Fischer, F. le Deist, S. Hacein-Bey-Abina et al., "Severe combined immunodeficiency. A model disease for molecular immunology and therapy," Immunological Reviews, vol. 203, pp. 98–109, 2005.

210. Davé UP, Akagi K, Tripathi R, et al. Murine leukemias with retroviral insertions at Lmo2 are predictive of the leukemias induced in SCID,X1 patients following retroviral gene therapy. *PloS Genet* 5:e1000491, 2009.

211. Mueller BU, Pizzo PA. Cancer in children with primary or secondary immunodeficiencies. *J Pediatr* 126:1-10, 1996.

212. Filipovich AH, Mathur A, Kamat D, et al. Primary immunodeficiencies: genetic risk factors for lymphoma. Cancer Res 52:5465-67, 1992.

213. Perlman S, Becker-Catania S, Gatti RA. Ataxia-telangiectasia: diagnosis and treatment. *Seminars in Pediatric Neurology* 10: 173-82, 2003.

214. Chun HH, Gatti RA. Ataxia-telangiectasia, an evolving phenotype. *DNA Repair* 3: 1187-96, 2004.

215. Ball LG, Xiao W. Molecular basis of ataxia telangiectasia and related diseases. *Acta Pharmacol Sin* 26:897-907, 2005.

216. Caldecott KW. DNA single-strand breaks and neurodegeneration. *DNA Repair (Amst)* 3:875-82, 2004.

217. Valentin-Vega YA, Maclean KH, Tait-Mulder J, et al. Mitochondrial dysfunction in ataxia-telangiectasia. *Blood* 119: 1490-500, 2012.

218. Harms C, Albrecht K, Harms U, et al. Phosphatidylinositol 3-Akt-kinase-dependent phosphorylation of p21(Waf1/Cip1) as a novel mechanism of neuroprotection by glucocorticoids. J Neurosci 2007; 27: 4562-571.

219. Amsterdam A, Tajima K, Sasson R. Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. *Biochem Pharmacol* 64:843-50, 2002.

220. Johnston MV. Plasticity in the developing brain: implications for rehabilitation. *Dev Disabil Res Rev* 15:94-101, 2009.

221. Griesemer AD, Sorenson EC, Hardy MA. The role of the thymus in tolerance. *Transplantation* 90:465-74, 2010.

222. Palmer E. Negative selection-clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* 3:383-91, 2003.

223. Hammerling GJ. Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen. *Immunol Rev* 122:47-67, 1991.

224. McGargill MA, Derbinski JM, Hogquist KA. Receptor editing in developing T cells. *Nat Immunol* 1:336-41, 2000.

225. Derbinski J, Gabler J, Brors B, et al. Promiscuous gene expression in thymic epithelial cells is regulated atmultiple levels. *J ExpMed* 202:33-45, 2005.

226. Gray D, Abramson J, Benoist C, et al. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J ExpMed* 204:2521-528, 2007.

227. Anderson MS, Veneziani ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395-401, 2002.

228. Gillard GO, Farr AG. Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J Immunol* 176:5815-24, 2006.

229. Mathis D, Benoist C. A decade of AIRE. *Nat Rev Immunol* 7:645-50, 2007.

230. Rooke R,Waltzinger C, Benoist C,MathisD. Targeted complementation ofMHCclass II deficiency by intrathymic delivery of recombinant adenoviruses. *Immunity* 7:123-34, 1997.

231. Klein L. Deadman walking: how thymocytes scan the medulla. *Nat Immunol* 10:809-11, 2009.

232. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6:331-37, 2005.

233. LiuH, LeungBP. CD4+CD25+ regulatory T cells in health and disease. *Clin Exp Pharmacol Physiol* 33:519-24, 2006.

234. Caton AJ, Cozzo C, Larkin J, et al. CD4(+) CD25(+) regulatory T cell selection. *Ann N Y Acad Sci* 1029:101-14, 2004.

235. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 6:1152-59, 2005.

236. Van Santen HM, Benoist C, Mathis D. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J ExpMed* 200:1221-30, 2004.

237. Foy TM, Page DM,Waldschmidt TJ, et al. An essential role for gp39, the ligand for CD40, in thymic selection. *J ExpMed* 182:1377-88, 1995.

238. Page DM, Roberts EM, Peschon JJ, et al. TNF receptor-deficient mice reveal striking differences between severalmodels of thymocyte negative selection. *J Immunol* 160:120-33, 1998.

239. Matzinger P,Guerder S.Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature* 338:74-6, 1989.

240. Kishimoto H, Sprent J. Several different cell surface molecules control negative selection of medullary thymocytes. *J ExpMed* 190:65-73, 1999.

241. Kishimoto H, Surth CD, Sprent J. A role for Fas in negative selection of thymocytes in vivo. *J Exp Med* 187:1427-38, 1998.

242. Nagamine K, Peterson P, Scott HS, et al. Positional cloning of the APECED gene. *Nat Genet* 17:393-8, 1997.

243. Mathew P, Crist WM, Furman WL. The use in cytokines in children. *Curr Opin Pediatr* 6:58-67, 1994.

244. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J Clin Endocrinol Metab* 91:2843-50, 2006.

245. Betterle C, Greggio NA, Volpato M. Autoimmune polyglandular syndrome type 1. *J Clin Endocr Metab* 83:1049-55, 1998.

246. Ahonen P, Myllarniemi S, Sipila I, et al. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med* 322:1829-36, 1990.

247. Tóth B, Wolff AS, Halász Z, et al. Novel sequence variation of AIRE and detection of interferonomega antibodies in early infancy. *Clin Endocrinol (Oxf)* 72:641-47, 2010.

248. Mathis D, Benoist C. Aire. *Annu Rev Immunol* 27:287-312, 2009.

249. Fischer A. Severe combined immunodeficiencies (SCID). *Clin Exp Immunol* 122:143-9, 2000.

250. Gu XXJ, Yue FY, Kovacs CM, et al. The role of cytokines which signal through the common g chain cytokine receptor in the reversal of HIV specific CD4+ and CD8+ T cell anergy. *Plos one* 2:e300, 2007.

251. Allderdice PW, Davis JG, Miller OJ, et al. The 13q-deletion syndrome. *Am J Hum Genet* 21:499–512, 1969.

252. Brown LY, Odent S, David V, et al. Holoprosencephaly due to mutations in ZIC2: Alanine tract expansion mutations may be caused by parental somatic recombination. *Hum Mol Genet* 10:791-96, 2001.

253. KiveläT, Tuppurainen K, Riikonen P, Vapalahti M. Retinoblastoma associated with chromosomal 13q14 deletion mosaicism. *Ophthalmology* 110:1983-88, 2003.

254. Brown S, Russo J, Chitayat D, Warburton D. The 13q- syndrome: The molecular definition of a critical deletion region in band 13q32. *Am J Hum Genet* 57:859-66, 1995.

SUMMARY

The immune system is an interactive network, including lymphoid organs, cells, humoral factors, and cytokines, which works synergistically in host defense from pathogens, such as viruses, bacteria, fungi, and parasites, and other foreign molecules. A disruption of this orchestrated process leads immunedeficiencies, allergy, autoimmune disease and tumors (3).

Primary immunodeficiencies (PIDs) are genetic disorders causing severe defects in different components of immune system. Nowadays, more than 200 well characterized genetic immunedeficiencies have been identified thanks to the advances in molecular genetics and immunology. PIDs are classified according to the component of the immune system that is primarily involved, including T, B, natural killer (NK) cells, phagocytic cells, and complement proteins. Within PIDs, the Severe combined immunodeficiency diseases (SCIDs) represent a spectrum of illnesses with similar clinical manifestations, which can be divided into several categories on the basis of the presence or absence of T cells, B cells and Natural Killer (NK) cells. These are relatively rare diseases, collectively occurring in 1:100.000 live births.

In this context, my PhD program has been focused to the study of some Immunological disorders, in order to identify "*New Scenarios in Pathogenesis and Therapeutic Approaches*".

Particularly, my research has been focused in the study functional role of FOXN1 transcription factor in the T-cell ontogeny, through the new strategy to develop an *in vitro* thymic organoid and the setting-up of the first phase of *in vitro* models generation to study this transcription factor by using reprogramming technology.

Moreover, my research effort has been devoted to characterization of the role of γc in cell cycle progression and survival of continuous and primary human malignant cell line, clarifying its involvement in leukemogenesis and the molecular mechanism by which this protein promotes tumor growth.

In addition, I gave a contribution to better define the mechanisms underlying the pathogenesis of forms of immunedeficiencies leading autoimmunity and cancer, respectively APECED and A-T.

Eventually, I participated to description of new clinical phenotype and novel genetic syndrome involving the immune system, opening new scenarios in the molecular mechanisms underlying the immune response.

These results obtained during my PhD program could be useful both in the clinical practice and in the basic research of immunedysregulation.

europass Curriculum Vitae

PERSONAL INFORMATION

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EDUCATION AND TRAINING

April 2011–Present

PhD student

Immunolgy Unit, Department of Pediatric, "Federico II" University of Naples, Naples (Italy)

Ph.D. student in *Human Reproduction, Development and Growth* with research projects entitled:

- "Characterization of the potential oncogenic role of γc by exploring its modulatory activity on genes related to growth signaling, cell cycle control and survival"

- "*In vitro* reprogramming of human WT and FOXN1-/- fibroblasts and differentiation in mTECs"

Supervisor Prof. Claudio Pignata

April 2013–July 2013

Training in methods of generation and maintenance of iPSCs

I-STEM / CECS, Genopole Campus 1 - 5, Rue Henri Desbrueres, Evry (France)

My task:

- Methods of genetic reprogramming, maintenance and characterization of induced Pluripotent Stem Cells (iPSCs) derived from skin fibroblasts. Supervisor Dr. Lina El Kassar

March 2008–June 2010

Master Degree in Medical Biotechnology (II level degree)

"Federico II" University of Naples - via Pansini, 5 - 80131, Naples (Italy)

Master Thesis entitled:“Functional role of CEACAM6 in $CD133^{+}$tumor stem cells in human model of colon cancer". Supervisor Dr. Rosa Di Noto

Vote: 110/110 laude

January 2005–March 2008

Bachelor in Biotechnology (I level degree)

"Federico II" University of Naples - via Pansini, 5 - 80131, Naples (Italy)

Bachelor Thesis entitled: "Methods for evaluation of expression of neuroprotective proteins promoting neuroprotection during preconditioning”. Supervisor Dr. Antonella Scorziello.

Vote: 108/110

WORK EXPERIENCE

April 2011–Present

PhD Student

Immunology Unit, Department of Pediatric, "Federico II" University of Naples - via Pansini, 5 - 80131, Naples (Italy)

My tasks:

- Generation and maintenance of inducend Pluripotent Stem Cells (iPSCs) from skin fibroblasts by reprogramming technology.

- Studing of the potential involvement of common gamma chain (γc) in hematopoietic malignancies by evaluating expression and function of genes γc-related involved in growth signaling, cell cycle control and survival.

- Writing of research articles and grants (see additional information).

September 2008–June 2010

Internship

Cell Culture Facility, CE.IN.GE - Advanced Biotechnologies Institute, Naples (Italy)

My task:

- Identification of new markers of Colon Cancer Stem Cells (CCSCs), by generation of *colon cancer sferoids* from human continuous colon cancer cell lines (GEO and CACO-2) and from human colon cancer biopsies.

September 2007–March 2008

Internship

Department of Neuroscience, "Federico II" University of Naples - via Pansini, 5 - 80131, Naples (Italy)

My task:

- Evaluation of transductional mechanisms responsible for the neuroprotective effect of nitric oxide (NO) during ischemic preconditioning (IPC).

PERSONAL SKILLS

Mother tongue(s): Italian

Other language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B1	B1	B1	B1	B2
French	A1	A1	A1	A1	A2

Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

- Predisposition to work in a team.
- Capacity to interact with other collegues and to work in multicultural environments.

Organisational / managerial skills

- Capacity to design a scientific project including the budgeting. (See the attached list of application to grant proposals)
- Capacity to administrate small budgets for the daily work in laboratory.
- Capacity to coordinate students in their practice in laboratory also by following them in the preparation of the their thesis.
- Capacity to contribute to the coordination and management of the scientific revision of articles required by international peer reviewed journals, as Cellular Immunology and Journal of Clinical Immunology.

Job-related skills

TECHNICAL SKILLS

Molecular biology:

- DNA and RNA extraction;
- electrophoretic separation of nucleic acid;
- cDNA synthesis by reverse transcription PCR;
- PCR;
- Transfection and RNA interference;
- Protein lysates and Western Blot.

Cellular biology:

- reprogramming of fibroblasts in iPSCs and stem cells culture;

- culture and cryopreservation of human and animal growing non-adherent and adherent cell lines;
- generation of fibroblast primary cell line from skin biopsy;
- cell generation of colon cancer sferoids from human continuous colon cancer cell lines;
- generation of single cell suspension from fresh human colon biopsies;
- B-cell immortalization by Epstein-Barr virus;
- cell proliferation assays;
- PBMC purification by ficoll;
- cytochemical staining;
- *in vivo* tumorigenic assay;
- cologenic assay;
- silencing by siRNA;
- knowledge of multiparametric flow cytometry analysis.

Computer skills

- Good knowledge of MS Windows, MacOS systems.
- Good knowledge of scientific softwares as: Oligo 4, Blast.
- Good knowledge of online databases of proteins (Swissprot/TrEMBL, PIR, UniProt, Prosite, ect), nucleotide sequences (GeneBank, EMBL,GeneCard) and browser UCSC Genome, mouse models (MGI, MPD, GXD, etc.).
- Use of Endnote 7.0 to format and add references to a manuscript.
- Use of Word, Excel, graphical softwares such as Power Point, Publisher, Photoshop for Mac and PC.

Driving licence B

Related document(s):

ADDITIONAL INFORMATION

Publications

1. Romano R., Palamaro L., Fusco A., Iannace L., Maio S., Vigliano I., Giardino G., Pignata C. From murine to human Nude/SCID: the thymus, T-cell development and the missing link. Clin Dev Immunol. 2012:467101, 2012.

2.Vigliano I., Palamaro L., Bianchino G., Fusco A., Vitiello L., Grieco V., **Romano R.**, Salvatore M., Pignata C. Role of the common γ chain in cell cycle progression of human malignant cell lines. Int Immunol. 24:159-167, 2012.

3.Palamaro L., Vigliano I., Giardino G., Cirillo E., Aloj G., **Romano R.**, Pignata C. SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. J Investig Allergol Clin Immunol. 22:67-70, 2012.

4.Cirillo E., **Romano R.**, Romano A., Giardino G., Durandy A., Nitsch L., Genesio R., Di Gregorio E., Cavalieri S., Abate G., Del Vecchio L., Brusco A., Pignata C. De novo 13q12.3q14.11 deletion involving BRCA2 gene in a patient with developmental delay, elevated IgM levels, transient ataxia and cerebellar hypoplasia, mimicking an A-T like phenotype. Am J Med Genet. 158 A:2571-76, 2012.

5.Palamaro L., Guarino V., Scalia G., Antonini D., De Falco L., Bianchino, G., Fusco A., **Romano R.**, Grieco V., Missero C., Del Vecchio L. Ambrosio L., Pignata C. Molecular signature of the T-cell commitment in an in vitro three-dimensional organoid mimicking the thymic microenvironment. J Clin Immunol. 32 (Suppl 1):S310, 2012 (Abstract).

6. Romano R., Ferrentino R., Pane L.S., Palamaro L., Fusco A., Marques J.G.,Sousa A.B.,de Sousa A.E., Ursini M.V.,BaldiniA., Pignata C. Preliminary steps of fibroblasts reprogramming to develop mTECs from control or FOXN1$^{-/-}$ fibroblasts. J Clin Immunol. 32 (Suppl 1):S332, 2012 (Abstract).

7. Cirillo E., **Romano R.**, Giardino G., Anne D., Maio F., Gallo V., Di Gregorio E., Cavalieri S., Abate G. Del Vecchio L., Brusco A., Pignata C. Elevated IgM levels in a patient with de novo 13q12.3q14.11 deletion , mimicking an A-T like phenotype. J Clin Immunol. 32:211-212, 2012(Abstract).

8. Palamaro L., Giardino G., Santamaria F., **Romano R.**, Fusco A., Montella S., Salerno M., Ursini M.V., Pignata C.Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and

very high IgE levels. Ital J Pediatr. 38:46, 2012.

9.Capalbo D., Giardino G., De Martino L., Palamaro L., **Romano R.**, Gallo V., Cirillo E., Salerno M., Pignata C. Genetic basis of altered central tolerance and autoimmune diseases: a lesson from AIRE mutations. Int Rev Immunol. 31:344-62, 2012.

10. GiardinoG., Fusco A., **Romano R.**, Gallo V., Maio F., Esposito T., Palamaro L., Parenti G., Salerno M.C., Vajro P., Pignata C. Betamethasone therapy in Ataxia-Telangiectasia: unraveling the rationale of this serendipitous observation on the basis of the pathogenesis. Eur J Neurol. 20:740-47, 2013.

11. Gallo V., Giardino G., Capalbo D., Palamaro L., **Romano R**., Santamaria F., Maio F., Salerno M., Vajro P., Pignata C. Alterations of the autoimmune regulator transcription factor and failure of central tolerance: APECED as a model. Expert Rev Clin Immunol. 9:43-51, 2013.

12.van de Vosse E., van Dissel J.T., Palamaro L., Giardino G., Santamaria F., **Romano R.**, Fusco A., Montella S., Salerno M., Ursini M.V., Pignata C. The R156H variation in IL-12Rbeta1 is not a mutation. Ital J Pediatr. 39:12, 2013.

13. Romano R., Palamaro L., Parenti G., Salerno M., Fusco A., Vajro P., Capalbo D., Ranieri B., Naddei R., Pignata C. Networking between γc and GH-R signaling in the control of cell growth. Curr Signal Transduct Ther. 8:67-73, 2013.

14. Palamaro L., Guarino V., Scalia G., AntoniniD., De FalcoL., BianchinoG., FuscoA., **RomanoR.**, GriecoV., MisseroC., Del VecchioL., AmbrosioL., Pignata C. Human skin-derived keratinocytes and fibroblasts co-cultured on 3D poly ε-caprolactone scaffold support *in vitro* HSCs differentiation into T-lineage committed cells. Int Immunol.25:703-14, 2013.

15. RomanoR., PalamaroL., FuscoA., GiardinoG., GalloV., Del VecchioL., PignataC. FOXN1: a master regulator gene of thymic epithelial development programme. Front Immunol. 2013 doi: 10.3389/fimmu.2013.00187.

16. Palamaro L., **Romano R.**, Fusco A., Giardino G., Gallo G., Pignata C. FOXN1 in cell development and human diseases. Int Rev Immunol. In press, 2013.

References

Prof. Claudio Pignata, M.D., PhD

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91030 Evry Cedex (France)

email: lelkassar@istem.fr

APPLICATIONS TO GRANT PROPOSALS

•SIRPED - Società Italiana di Ricerca Pediatrica 2011, with a project entitled: “Modulation of molecular mechanism implicated in the high predisposition to infection and cancer in children with Ataxia-Teleangiectasia”.

•Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2010-11, with a project entitled: “Sviluppo di approcci innovativi diagnostici e terapeutici per le immunodeficienze primitive”.

•Futuro in Ricerca - Call for MIUR Application 2012, with a project entitled: “Studio dei meccanismi che regolano l'omeostasi dei linfociti B e T nelle Immunodeficienze Primitive: difetti primitivi a carico dei linfociti T e l'Immunodeficienza Comune Variabile (CVID) come modelli”.

•Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2012, with a project entitled: “Sviluppo di approcci innovativi diagnostici e terapeutici per le

immunodeficienze primitive"

•Futuro in Ricerca - Call for MIUR Application 2013, with a project entitled: "Characterization of the potential oncogenic role of c by exploring its modulatory activity on genes related to growth signaling, cell cycle control and survival, andthrough its post-transcriptional miRNAs-mediated regulation".

•Telethon Grant Proposals - Call for Applications 2010, with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its pathogenic implication in Ataxia Telangiectasia: potential modulatory effect of Betamethasone".

MEETING ABSTRACTS AND COMMUNICATIONS

•Palamaro L., Guarino V., Scalia G., Antonini D., De Falco L., Bianchino G., Fusco A., **Romano R.**, Grieco V., Missero C., Del Vecchio L., Ambrosio L., Pignata C. Molecular signature if the T-cell commitment in an in vitro three-dimentional organoid mimicking the thymic microenvironment. 15th Biennial Meeting of the European Society for Immunedeficiencies, Florence 3-6 October, 2012 (Poster).

•**Romano R.**, Ferrentino R., Pane L.S., Palamaro L., Fusco A., Marques J.G., Sousa A.B., de Sousa A.E., Ursini M.V., Baldini A., Pignata C. Preliminary steps of fibroblasts reprogramming to develop mTECs from control or FOXN1-/- fibroblasts. 15th Biennial Meeting of the European Society for Immunedeficiencies, Florence 3-6 October, 2012 (Poster).

•Cirillo E., **Romano R.**, Giardino G., Anne D., Maio F., Gallo V., Di Gregorio E., Cavalieri S., Abate G. Del Vecchio L., Brusco A., Pignata C. Elevated IgM levels in a patient with de novo 13q12.3q14.11 deletion , mimicking an A-T like phenotype. 15th Biennial Meeting of the European Society for Immunedeficiencies, Florence 3-6 October, 2012 (Poster).

1. APPLICATIONS TO GRANT PROPOSALS

(Dr Rosa Romano)

1. SIRPED - Società Italiana di Ricerca Pediatrica 2011, with a project entitled: "Modulation of molecular mechanism implicated in the high predisposition to infection and cancer in children with Ataxia-Teleangiectasia".

2. PRIN - Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2010-11, with a project entitled: "Sviluppo di approcci innovativi diagnostici e terapeutici per le immunodeficienze primitive".

3. Futuro in Ricerca - Call for MIUR Application 2012, with a project entitled: "Study of the mechanisms that regulate B and T cell homeostasis in Primary Immunodefiencies: primary T cell defects and Common Variable Immunodeficiency (CVID)as models".

4. PRIN - Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2012, with a project entitled: "Sviluppo di approcci innovativi diagnostici e terapeutici per le immunodeficienze primitive".

5. Futuro in Ricerca - Call for MIUR Application 2013, with a project entitled: "Characterization of the potential oncogenic role of γc by exploring its modulatory activity on genes related to growth signaling, cell cycle control and survival, and through its post-transcriptional miRNAs-mediated regulation".

6. Telethon Grant Proposals - Call for Applications 2010, with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its pathogenic implication in Ataxia Telangiectasia: potential modulatory effect of Betamethasone".

2. SCIENTIFIC PRODUCTION

(Dr Rosa Romano)

LIST OF PUBLICATIONS

1. **Romano R.**, Palamaro L., Fusco A., Iannace L., Maio S., Vigliano I., Giardino G., Pignata C. From murine to human Nude/SCID: the thymus, T-cell development and the missing link. Clin Dev Immunol. 2012:467101, 2012.

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13. Fusco A., Panico L., Gorrese M., Bianchino G., Barone MV., Grieco V., Vitiello L., D'Assante R., **Romano R.**, Palamaro L., Scalia G., Del Vecchio L., Pignata C.

Molecular Evidence for a Thymus-Independent Partial T Cell Development in a FOXN1(-/-) Athymic Human Fetus. PLoS One. 8:e81786, 2013.

14. Palamaro L., **Romano R.**, Fusco A., Giardino G., Gallo G., Pignata C. FOXN1 in cell development and human diseases. Int Rev Immunol. In press, 2014.

15. Pignata C. and **Romano R.** In this issue: FOX genes and the immune response. Int Rev Immunol. In press, 2014.

MEETING ABSTRACTS

1. Palamaro L., Guarino V., Scalia G., Antonini D., De Falco L., Bianchino G., Fusco A., **Romano R.**, Grieco V., Missero C., Del Vecchio L., Ambrosio L., Pignata C. Molecular signature if the T-cell commitment in an in vitro three-dimentional organoid mimicking the thymic microenvironment. 15th Biennial Meeting of the European Society for Immunedeficiencies, Florence 3-6 October, 2012. J Clin Immunol. 32:310, 2012 (Poster).

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