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NEW INSIGHTS AND UNSOLVED ISSUES IN CONGENITAL IMMUNODEFICIENCIES

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BACKGROUND AND AIMS

Primary immunodeficiencies comprise more than 200 different disorders that affect the development and the functions of the immune system. In most cases primary immunodeficiencies are monogenic disorders that follow a simple mendelian inheritance. Primary immunodeficiencies are rare and have an overall prevalence of approximately 1:10.000 live births and are classified according to the component of the immune system that is primarily involved.

Primary immunodeficiencies are characterized by increased susceptibility to severe infections with distinctive susceptibility to various types of pathogens depending on the nature of the immune defect. The study of primary immunodeficiencies has led to better understanding the mechanisms that are involved in adaptive immune responses and innate immunity. Primary immunodeficiencies 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 (**Figure 1**) (1).

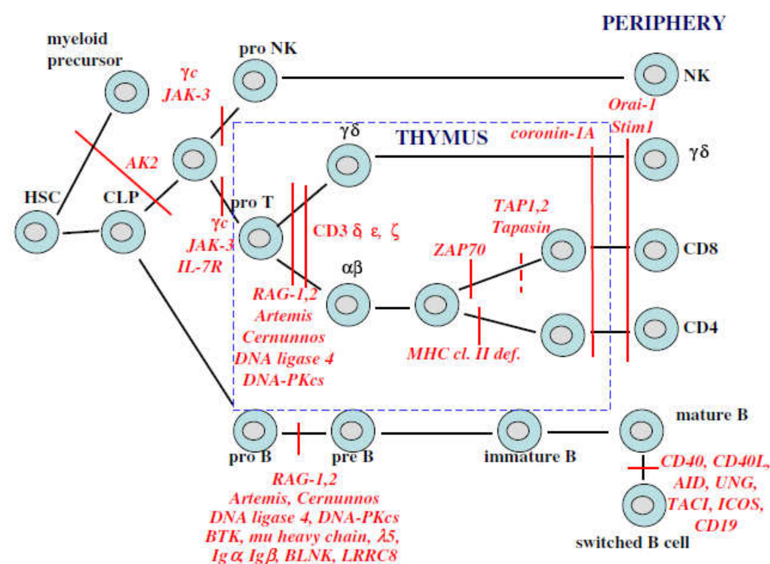

Figure 1. Genetic defects in Primary Immunodeficiencies

Severe combined immunodeficiencies (SCIDs) represent a spectrum of illnesses with similar clinical manifestations, which can be subdivided 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 (2-5). Without effective treatment, patients typically die of opportunistic infections before 1 year of age. SCID can be cured by bone marrow transplantation in most instances (4). Various mechanisms of these diseases have been described. 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 (6, 7). 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 (8, 9). Deficiency in expression or function of the γ common (γc) cytokine receptor subunit shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 causes the X-linked form of SCID (X-SCID), characterized by the complete absence of both T and NK lymphocytes (10). Deficiency in JAK3, which is normally associated with the cytoplasmic region of γc, results in an identical phenotype (10). Deficiency in either RAG1 or RAG2 (the lymphoid-specific recombination-initiating elements) or Artemis (a factor involved in the nonhomologous end-joining repair pathway) leads to defective V(D)J rearrangements (5) and thereby thymocyte and pre-B cell death. Defective pre-TCR and TCR signaling was also described. Pure T-cell deficiencies are caused by defects in either a CD3 subunit (such as CD3δ, CD3ε or CD3ζ) (5) or in the CD45 tyrosine phosphatase (11), key proteins involved in pre-TCR and/or TCR signaling at the positive selection stage. Other T-cell immunodeficiencies in the SCID group, such as ZAP-70 deficiency (12), CD3γ deficiency (13), HLA class II expression deficiency (14), purine nucleoside phosphorylase deficiency (15), ligase IV or Cernunnos deficiency (16) and Omenn syndrome (17) were included.

Most of the genes, whose alterations underlie SCIDs, are selectively expressed in hematopoietic cells. In the 1996, for the first time, a novel form of SCID was

described, defined as Nude/SCID syndrome (18). This syndrome represents the first example of SCID not primarily related to an abnormality of the hematopoietic cell. The disease is due to a molecular alteration of the transcription factor FOXN1, which is selectively expressed in thymic and cutaneous epithelia (19).

Some forms of primary immunodeficiencies show a more complex phenotype in which immune dysregulation is only one of multiple components of the disease phenotype. Several immunodeficiencies are caused by defects in mechanisms of DNA breaks repair. Ataxia-telangiectasia is an autosomal recessive disease caused by mutations of the ataxia-telangiectasia mutated gene (ATM). Patients with ataxia-telangiectasia have ataxia, ocular telangiectasia, increased risk of infections and tumors.

This thesis reports the results obtained during my PhD course in “Human Reproduction, Development and Growth” (XXIV Cycle) from 2008 to 2011.

My PhD programme has been focused in the study of the following lines of research:

- ✓ Role of γc in cell cycle progression, strongly related to its cellular amount and Growth Hormone Receptor (GH-R) signaling, defining the basis of the physiological interaction between endocrine and immune systems;
- ✓ Molecular and clinical characterization of the human Nude/SCID phenotype and study of functional role of transcription factor FOXN1 in the T-cell ontogeny and in the nervous central system development;
- ✓ Effects of steroid treatment in patients affected with Ataxia-Telangiectasia;
- ✓ Immunodeficiencies associated with unidentified molecular defects.

CHAPTER I

"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 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 an immune system can be reconstituted. The best current treatment for X-SCID is bone marrow transplantation (BMT) from an HLA-matched related donor. This therapeutic approach 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. Two separate trials for X-SCID have shown the clinical feasibility of introducing a therapeutic gene into hematopoietic stem cells (20). The deficiency was restored and lymphocyte development was no longer blocked (21-23), but the occurrence of leukemia in five patients in the trials has emphasized that insertional mutagenesis and its oncogenic consequence is an unexpectedly frequent adverse effect of gamma-retroviral gene transfer technology.

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.

§1.1 Biology of the γc transducing element

IL-2RG encodes the γc of the IL-2 receptor. The γc gene, localized to chromosome Xq13, encodes a transmembrane protein which is a transducing element shared by the cytokine receptor superfamily (2, 24).

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 (25). The cytokine receptor superfamily is the largest family, in which the receptors for IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) contain the common gp130 (26, 27), the receptors for IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) share the common beta subunit (28), whereas the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 and GH-R share the common γc element (25).

The γc is expressed in lymphocytes and also in other cell types. It is now clear that γc cytokines regulate several aspects of immune activation; they play an important role in supporting survival, proliferation and effector functions of activated immune cells. Clearly, regulation of cell survival and cell apoptosis is a delicate teamwork and a balanced act of all γc-dependent cytokines is of central importance (**Figure 2**) (29).

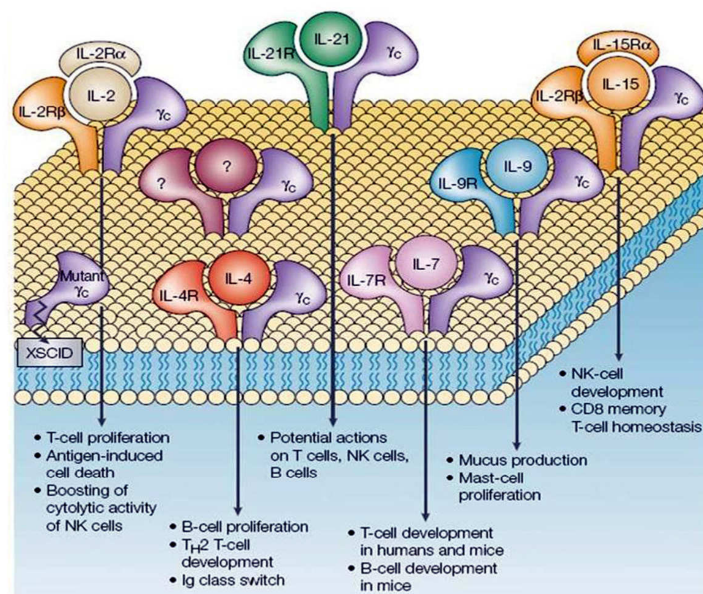

Figure 2. The γc dependent cytokines

The biological effects of cytokines are mediated through interaction with specific receptors, this leads to phosphorylation of intracellular proteins. Members of the cytokine receptor superfamily do not have intrinsic kinase activity, but recruit intracellular protein kinases following interaction with their ligands (30). 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 IL-2R superfamily physically are associated with JAK1 and JAK3 (31, 32). In the IL-2R, JAK1 interacts with the serine region of the β subunit, whereas the 48 C-terminal residues of the γc are required to bind JAK3. Both regions of the IL-2R chains are critical for JAK activation and signal transduction. Following cytokine-cytokine receptor interaction and dimerization of the cytoplasmic tails of the cytokine receptor chains, the JAKs are brought into close proximity and may cross-phosphorylate each other. Several signaling pathways are elicited by JAK1/JAK3 activation in members of the cytokine receptor superfamily (33).

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 and results in the activation of Raf-1 mitogen-activated protein kinase (MAPK) and eventually in the induction of immediate-early genes (*c-fos*, *c-jun*) (34).

Second, JAKs may bind and phosphorylate insulin receptor substrates (IRSs). JAK activation by γc dependent cytokines results in phosphorylation of IRS-1, whereas evidence of JAK-dependent tyrosine phosphorylation of IRS-2/4 has so far been obtained only for IL-2, IL-4, IL-7 and IL-15 (35). Once activated, IRS may bind the SH2 domain of the p85 subunit of phosphatidylinositol-3-kinase (PI3K) and the catalytic activity of the p 110 subunit of PI3K is eventually

elicited. In addition to promoting PI3K activation, IRS may recruit Grb2 and thus amplify the Ras/raf-1 signaling pathway (36-38).

A third essential event of the JAK signaling pathway is the phosphorylation of the class of transcription factors known as signal transducers and activators of transcription (STATs) (39). The STATs factors comprise a diverse group of cytoplasmic proteins that are involved in several functions, such as regulation of the expression of effector genes, cell differentiation, survival and apoptosis (40). So far, seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 have been characterized (40). The STATs contain a tyrosine residue that may undergo JAK-mediated phosphorylation and they also contain SH2 and SH3 domains. 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, STAT-STAT homo- or heterodimerization occurs, with the SH2 domain of one STAT molecule binding 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. However, three crucial lymphocyte growth factors, IL-2, IL-7 and IL-15 activate STAT3 and STAT5 (41).

Following dimerization, STATs translocate into the nucleus, where they bind to consensus sequences in the enhancer elements of the promoter regions of target genes and favor gene transcription (**Figure 3)**.

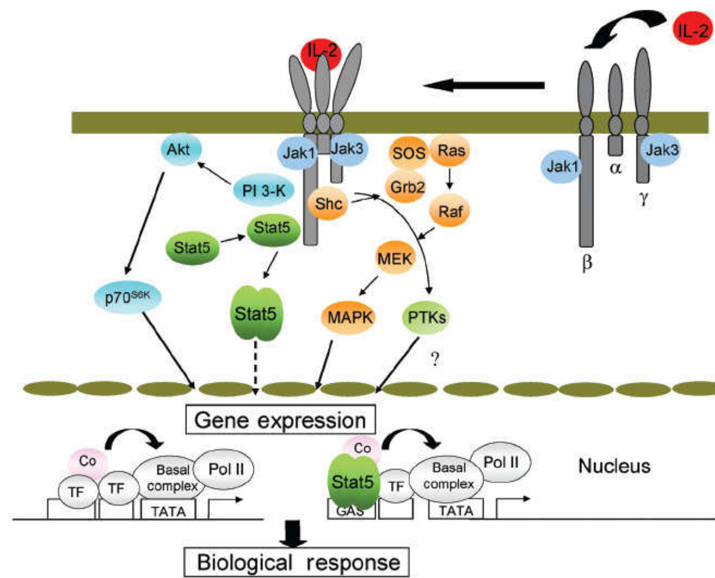

Figure 3. The γc signaling transduction

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 (42). Previously, it has been reported on a patient affected with X-SCID, short stature and peripheral GH hyporesponsiveness, an abnormal protein phosphorylation that normally occur following GH-R stimulation (43). 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) (44). GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function.

The GH-R was the first member of the cytokine receptor superfamily to be cloned (45). Similarly to other members of the cytokine receptor superfamily, it consists of a transmembrane protein that contains two motifs and an extracellular domain (46). GH-R lacks intrinsic kinase activity and signal transduction is mediated by receptor-associated cytoplasmic tyrosine kinases. A prominent role is played by the JAK2 that associates to the GH-R cytoplasmic domain (47). After phosphorylation of JAK2, the receptor itself and several intracytoplasmic

molecules are promptly phosphorylated on tyrosine residues. Further signaling proteins recruited to GH-R/JAK2 complex and/or activated in response to GH include: Shc proteins that presumably lead to the activation of MAPK pathway (48), insulin receptor substrates that has been implicated in the activation of PI3K and the kinase AKT/protein kinase (PK) B (49, 50), phospholipases that lead to formation of diacylglycerol and activation of PKC and a variety of proteins that are involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck (51, 52). This process ultimately results in the activation of STAT family members. STATs proteins dimerize and translocate into the nucleus, where they bind to specific DNA responsive elements of GH target genes, eventually inducing the activation of gene transcript (53). The duration of GH-activated signals is a key factor in relationship to the biological actions of the hormone. Removal of cell surface GH-R by endocytosis is an early step in the termination of GH-dependent signaling (54). Furthermore, suppressors of cytokine signaling (SOCS) proteins act as negative regulators of the JAK/STAT signal cascade (55). Moreover, several studies documented that there are at least three different phosphatases involved in the specific down-regulation of GH-R signaling: SH2 domain-containing protein-tyrosine phosphatase (SHP)-1, tyrosine-protein phosphatase (PTP)-1B and PTP-H1 (40).

Thus, it is clear that STAT5 proteins, in particular, are strongly correlated with some oncogenic events, such as proliferation and apoptosis (56); so, the therapeutic inhibition of these transcription factors may be proven helpful for those diseases characterized by an alteration of cell growth homeostasis. Moreover, the development of selective inhibitors of STAT activation may be a promising area in the field of novel anticancer therapeutics (57).

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.

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Review

γ Chain transducing element: A shared pathway between endocrine and immune system

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ABSTRACT

Several molecules, involved in the intracellular communication network, have been identified as the cause of primary immunodeficiencies. In most cases, these molecules are exclusively expressed in hematopoietic cells, being involved in cell development and/or functionality of terminal differentiated cells of immune system. In the case of γc, the abundance of the protein suggests a potential pleiotropic effect of the molecule. 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. It has been demonstrated a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, supporting the hypothesis of an interplay between endocrine and immune system. The evidence that different receptors share a few molecules may certainly lead to a better knowledge on the mechanism of coordination and integration of several pathways implicated in the control of cell growth and proliferation under physiological or pathogenic conditions. 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.

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1. 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 interleukin (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) [2]. SCIDs are a group of rare primary immunodeficiencies (PID), distinct in either the clinical and immunological phenotype or the pathogenetic mechanism. X-SCID is the most common form of SCID, accounting for approximately half of the cases of SCID and is the main form of $T^-B^+NK^-$, in which T cells and natural killer (NK) cells are absent or profoundly diminished in number, whereas B-cells are normal in number even though not functional. 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 several cytokines. Additional molecules, involved in the intracellular communication network, have been identified as responsible of peculiar forms of SCID, including IL-7R and Janus kinase (JAK) 3 [3,4]. In most cases, these molecules are exclusively expressed in hematopoietic cells, being involved in cell development and/or functionality of terminal differentiated cells of immune system. The abundance of γc is much higher than the aliquot expressed in hematopoietic cells, thus leading to hypothesize a pleiotropic effect of the molecule [5,6].

It is noteworthy that 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 [7]. There is evidence documenting that immune cells express growth hormone receptor (GH-R) [8]. It has also recently been shown a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, suggesting an interplay between endocrine and immune systems [8]. GH-R can promote cell cycle progression of lymphoid cells and of a wide variety of other cells. Indeed, recently, it has been documented a direct involvement of γc in self-sufficient growth and GH induced proliferation in a concentration dependent manner of the molecule [9]. GH-R signaling apparatus also involves potent mitogenic molecules such as signal transducers and activators of transcription (STATs) that play a role in cell proliferation [10].

This review will focus on the relationship between different receptors that share common transducing elements and on the potential clinical implications of such still poorly understood interactions.

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2. γ Chain: a shared component of several cytokine receptors

The cytokines are soluble elements that control the immune and the hematopoietic system [11]. In particular, cytokines and growth factors transmit signals through specific cell-surface receptors to the nucleus by activating intracytoplasmic signaling molecules that ultimately result in the activation of transcription factors. Their functions are due to the various receptors expressed on multiple target cells [12] and their rule is closely dependent on the recognized targets.

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 [13]. The cytokine receptor superfamily is the largest family, in which the receptors for IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) contain the common gp130 [14,15], the receptors for IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) share the common beta subunit [16], whereas the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 share the γc element [13].

The characterization of cytokine-activated genes, including genes regulated by γc-dependent cytokines, has long been an area of considerable interest, leading to define a prominent role for various immunological functions. An unanimously accepted series of evidence indicates that the γc-dependent cytokines control the immune response at different as well as overlapping checkpoints [17]. Most of the information so far available on the role of γc came out from studies on X-SCID in humans and in mice carrying mutations in the γc gene [1,11,18].

IL-2 is a growth factor, regulating the proliferation and apoptosis of activated T cells [19]. Moreover, IL-2 promotes NK cell cytolytic activity and immunoglobulin production by B cells [20]. IL-4 is required for the development and function of T helper 2 (Th2) cells and has an important role in allergy and immunoglobulin class switching [21]. Indeed, a role for IL-4 in B-cell Ig class-switch to IgG1 and IgE has been described [22]. IL-7 regulates lymphocyte development and homeostasis and exerts effects on both T- and B-cell biology [23–25]. In addition, IL-7 is well known for its potent role as a lymphocyte survival factor [26,27]. IL-9 is produced by a subset of activated $CD4^+$ T cells [28] and induces the activation of epithelial cells, B cells, eosinophils and mast cells [28], but its role in T cell biology remains unclear. IL-9 deficient mice have also been generated and, in these animals, the lymphoid compartment develops normally. However, these mice exhibit excessive mucus production and mast cell proliferation [29]. Interestingly, IL-9 transgenic mice develop thymic lymphomas, consistently with the presence of IL-9R in the thymus and with the ability of thymocytes to respond to IL-9 [30]. IL-15 is essential for the development of NK cells, in that mice deficient in IL-15 lack NK cells [31,32]. Furthermore, IL-15 is essential for the homeostatic proliferation of memory $CD8^+$ T cells [27]. IL-21 is the most recently described member of the γc family [33] and it has broad actions that include promoting the terminal differentiation of B cells to plasma cells, cooperating with IL-7 or IL-15 to drive the expansion of $CD8^+$ T cell populations and acting as a pro-apoptotic factor for NK cells and incompletely activated B cells [33].

In addition, γc-dependent cytokines also play an important role in supporting cell survival of activated immune cells. Clearly, regulation of cell survival and cell apoptosis is a delicate teamwork and a balanced action of all γc-dependent cytokines is of central importance. Thus, an abnormality of either one of them can have a profound impact on the homeostasis of the immune system.

Overall, in spite of the diversity of the numerous functions related to γc-containing receptors, the most important biologic effect shared between these cytokines receptors seems to be the mitogenic effect [34].

3. Signal transduction through GH receptor

GH participates to an integrated network with other mitogenic factors, as 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, all of them being involved in tissue growth. GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function. The several functions are mediated by an array of distinct signals triggered by an individual receptor, thus implying that diverse signaling pathways may be activated separately and in the context of a function specific coordinating network [35]. The GH-R was the first member of the cytokine receptor superfamily to be cloned [36]. Similarly to other members of the cytokine receptor superfamily, it consists of a transmembrane protein that contains two motifs and an extracellular domain [37]. Like other members of the family, GH-R lacks intrinsic kinase activity and signal transduction is mediated by receptor associated cytoplasmic tyrosine kinases.

A prominent role is played by the JAK2 that associates to the GH-R cytoplasmic domain [38]. After 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 [39]; insulin receptor substrates that has been implicated in the activation of phosphatidylinositol-3-kinase (PI3K) and the kinase AKT/protein kinase (PK) B [40,41]; phospholipases that lead to formation of diacylglycerol and activation of PKC and a variety of proteins that are involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck [42,43]. This process ultimately results in the activation of STAT family members. STATs proteins dimerize and translocate into the nucleus, where they bind to specific DNA responsive elements of GH target genes, eventually inducing the activation of gene transcript [44].

The duration of GH-activated signals is a key factor in relationship to the biological actions of the hormone. Removal of cell surface GH-R by endocytosis is an early step in the termination of GH-dependent signalling [45]. Furthermore, suppressors of cytokine signaling (SOCS) proteins act as negative regulators of the main cytokine-activated signaling pathway, the JAK/STAT signal cascade [46]. Moreover, several studies documented that there are at least three different phosphatases involved in the specific down-regulation of GH-R signaling: SH2 domain-containing protein-tyrosine phosphatase (SHP)-1; tyrosine-protein phosphatase (PTP)-1B and PTP-H1 [47].

This knowledge would help understand tissue specificity of GH action and would allow devise strategies to enhance individual functions of GH. Thus, pharmacological targeting of specific negative regulators of GH signaling would have a remarkable potential to enhance the beneficial effects of GH [46].

4. JAK/STAT signaling

Following the interaction of cytokines and growth factors with their receptors [48,49], the tyrosine kinases bind and phosphorylate the cytoplasmic tail of the receptors [50]. In this process, the JAK family members play a prominent role. Thus far, four distinct members of the JAK family are known in humans: JAK1, JAK2, JAK3 and Tyk2.

Following receptor dimerization, JAKs activate downstream molecules through three different transduction mechanisms. First, the phosphorylated cytokine receptor may associate with the adaptor Shc, which is itself phosphorylated and binds to Grb2 [51]. Grb2 may thus anchor to the Ras guanine nucleotide exchanging factor (Sos) [52]. Membrane translocation of the Grb2/Sos complex catalyzes the conversion of inactive GDP-bound Ras to the active GTP-bound state [53]. This results in the activation of Raf-1, MAPK and eventually in the induction of immediate-early genes (c-fos and c-jun) [54]. Second, JAKs may bind and phosphorylate insulin receptor substrates (IRSs). Indeed, JAK activation by interleukins results in the phosphorylation of IRS-1 [55]. Once activated, IRS may bind the PI3K. In addition to promoting PI3K activation, tyrosine phosphorylated IRS may recruit Grb2 and thus amplify the Ras/Raf-1 signaling pathway [56].

A third essential component of the JAK signaling pathway is the phosphorylation of the class of transcription factors known as STAT molecules [57]. The STATs factors comprise a diverse group of cytoplasmic proteins that are involved in several functions, such as regulation of the expression of effector genes, cell differentiation, survival and apoptosis [47]. So far, seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 have been characterized [47]. The STATs contain a tyrosine residue that may undergo JAK-mediated phosphorylation, and they also contain SRC homology (SH) 2 and SH3 domains. Following 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 [57]. Following dimerization, STATs translocate to the nucleus, where they bind to consensus sequences in the enhancer elements of the promoter regions of target genes and favour gene transcription. Gene accessibility to STAT binding is another mechanism through which specific responses to distinct cytokines are obtained. It has been suggested that JAK-dependent STAT activation is more crucial to cell differentiation than to proliferation. 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.

STAT proteins are essential regulators of cell proliferation, differentiation and survival in different cellular contexts, thus revealing their critical role in malignant transformation. STATs molecules have been demonstrated to directly participate in tumor development and progression [58]. Knockout studies have also highlighted the function of STAT proteins in the development and function of the immune system and of their roles in maintaining peripheral immune tolerance and tumour surveillance.

STATs are activated by a number of cytokines, including interferons and interleukins, as well as growth factors and hormones. STAT1 is inducible by interferon (IFN)-α/β and IFN-γ and is involved in anti-viral and anti-bacterial response, in growth inhibition, apoptosis and tumor suppression [47]. STAT3 is mainly activated by IL-6 and epidermal growth factor (EGF) and is involved in mitogenesis, survival, anti-apoptosis and oncogenesis [59]. 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 [47]. STAT6 molecule is activated by IL-4 and participates in Th2 development [58]. STAT5a and STAT5b are involved in prolactin and growth hormone signaling. STAT3 and STAT5, have been demonstrated to directly participate in tumor development and progression [60,61].

STATs participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators such as Bcl-x_L, Mcl-1, cyclins D1/D2, and c-Myc [62–64]. Moreover, tumor cells possessing activated STAT3 or STAT5 are predicted to be resistant to chemotherapeutic agents that may utilize similar apoptotic pathways. It has been clearly documented that inhibition of constitutively active STATs results in growth inhibition and induction of apoptosis in tumor cells [61,65].

Recent studies have shown that JAK/STAT signaling can be regulated through distinct mechanisms. Down-regulation of cytokine-JAK/STAT signaling is important for homeostasis and the prevention of chronic inflammation or autoimmunity. Moreover, also constitutive inhibitory pathways and inducible mechanisms have been described. Constitutive inhibitory mechanisms include the proteolysis, dephosphorylation and interaction with inhibitory molecules termed protein inhibitors of activated STATs (PIAS) [66]. Regulated or inducible inhibitory mechanisms have been identified. The receptor expression is down-regulated, through the induction of inhibitory molecules termed SOCS proteins and by rapid MAPK or PKC-dependent modification of pre-existing signaling components.

A potential novel pharmacological strategy may be to develop specific drugs that can specifically target the JAK-STAT regulators or the motifs implicated in such intermolecular interactions.

5. Networking between γc and GH-R signaling: atypical patients as "nature experiments"

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 [8].

Recent evidence indicates that silencing of γc induces a considerable decrease of the protein amount in lymphoblastoid cell lines that results in a reduction of self-sufficient growth in a concentration dependent manner along with a decrease of the response of lymphoblastoid cells to GH-induced proliferation and STAT5 subcellular redistribution following GH-R perturbation [9]. 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 documented [9]. Taken together, these data imply a direct involvement of γc in the control of cell cycle progression. Previously, it has been reported on a patient affected with X-SCID, short stature and peripheral GH hyporesponsiveness, an abnormal protein phosphorylation that normally occur following GH-R stimulation [67]. 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 insulin growth factor I (IGF-I) [68].

This would also imply that haematopoietic-derived cells represent an important source of those intermediate molecules that play a role in the GH-R functionality.

6. Clinical implications of alterations of GH-R/IGF-I axis in immune response and abnormal cell growth

The effects of GH on growth are mostly mediated by intermediate factors [69]. GH upon binding to its receptor initiates the signaling cascade, which culminates in the regulation of multiple genes, including IGF-I and its major binding protein, the IGF binding protein-3 (IGFBP-3). IGF-I with the IGFBP-3 and the acid labile subunit (ALS) is released into the circulation as a ternary complex [70].

Evidence supports a role for GH acting as a cytokine in the immune system under conditions of stress, counteracting immunosuppression by glucocorticoids [71]. Lymphoid cells express the

GH-R and GH can be produced by immune tissues, suggesting an autocrine/paracrine mode of action of GH. Moreover, GH can, directly or indirectly through the production of IGF-I, promote cell cycle progression and prevent apoptosis of lymphoid cells and of a wide variety of other cells, as well. It has been demonstrated that both GH and IGF-I are able to promote cell survival and proliferation through independent different pathways, thus indicating a potential function related specificity of the individual pathway [72]. It has been suggested that GH treatment may partially protect immune cells against apoptosis induced by stress conditions and deregulated expression of GH may participate to the development of malignancies of immune cells, such as leukemias or lymphomas [71]. Moreover, IGF-I induces a number of biologic effects, as induction of cell growth through the activation of cell cycle machinery, maintenance of cell survival by acting on the Bcl family members and induction of cellular differentiation through still poorly characterized mechanisms [73]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factor [74]. Components of the IGF-I system may play a key role in the deregulation of cell cycling or apoptosis in tumor growth [75].

As for the relationship between the GH/IGF-I axis and the risk of developing cancer, no conclusive data are available. There is evidence indicating that the GH/IGF-I axis has a role in the development of cancer through the regulation of cell proliferation, differentiation and apoptosis [76]. In particular, the association between circulating IGF-I and IGFBP-3 concentrations and the risk of developing cancer was documented [77]. 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 supposed to also have antiproliferative and proapoptotic effects, thus interfering with tumor growth [77]. Moreover, in the transformed cell, there are several data showing that IGF-I-R regulates cancer cell proliferation, survival and metastasis [78]. Differently from IGF-I and IGFBP3, the involvement of GH in the physiopathology of cancer is an open issue.

Progress in defining the pathogenic implications of 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. Therefore careful attention to future clinical applications of these therapeutic targeting in combination with chemotherapy will be necessary [79].

The GH-R signaling apparatus also involves potent mitogenic molecules such as γc and STATs that play a role in the cell proliferation and, in general, in cell homeostasis. It should be noted that overexpression of γc, in patients treated with gene therapy for X-SCID, resulted in lymphoproliferation, as a consequence of insertional oncogenesis in LMO2 oncogene [80,81]. However, the insertional mutagenesis was not found in all patients who developed the lymphoproliferative disorder, thus suggesting a direct involvement of γc in self-sufficient growth and activation induced proliferation [9].

Clinical studies have greatly contributed in defining that STATs are key molecules in GH-R signaling and in understanding the mechanisms by which GH activates genes that lead to its physiological functions. In particular, STAT5b appears to be involved in GH mediated IGF-I gene transcription and production of IGF-I and in transcription and production of IGFBP-3 and the ALS as well [82]. In patients carrying mutations of STAT5b gene a marked reduction of the GH-dependent peptides IGF-I, IGFBP-3 and ALS has been observed [83–85] while basal and stimulated GH concentrations were either normal or increased. These patients were characterized by growth failure and immunodeficiency [83–87].

However, the relationship between endocrine and immune dysfunctions in patients with STAT5b alterations are not yet completely defined [88]. Certainly, STAT5b seems a shared component between signaling pathways implicated in both immunological and endocrine functions. Even though, any cytokines, as IL-2, IL-7, IL-21 and IFN-γ, can activate STAT5b. Most of the current knowledge about the biological function of STAT family members has been achieved through disruption approaches and studies of knock-out (KO) mice [48]. In particular, it has been supposed that STAT5a/b would have very fundamental functions in regulating cell growth. Indeed, STAT5a and STAT5b KO mice have a most important failure of several responses associated with growth hormone secretion. In particular, the deletion of STAT5b alone gives a phenotype analogous to that observed in GH-R deficient mice, resulting in a failure of postnatal growth.

The role of STAT5 in the immune system has also been extensively analyzed. Splenocytes from STAT5a KO mice have a partial defect in anti-CD3-induced proliferation that can be overcome by high doses of IL-2 [89]. Moreover, also splenocytes from STAT5b KO mice exhibit greatly diminished proliferation in response to IL-2 and IL-15. Moreover, STAT5b is essential for potent NK cell-mediated proliferation and cytolytic activity [90]. However, an impaired proliferation of peripheral T lymphocytes has been observed in STAT 5a/b KO mice, even if this feature is, probably, due to a defect in the cell cycle entry rather than to a decreasing of IL-2 receptor expression. In fact, while lymphopoiesis is normal, T cells from double KO mice show a marked failure to undergo cell cycle progression and a diminished expression of proteins fundamental for proliferation [91].

Thus, it is clear that STAT5 proteins are strongly correlated with some oncogenic events, such as proliferation and apoptosis [91]; so, the therapeutic inhibition of these transcription factors may be proven helpful for those diseases characterized by an alteration of cell growth homeostasis. Moreover, the development of selective inhibitors of STAT activation may be a promising area in the field of novel anticancer therapeutics [61].

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.

Conflict of interest

None.

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§1.2 The γc provides spontaneous or induced cell proliferation

The intrinsic property of γc in cell cycle progression has been long debated. In fact, although gene therapy trials have been proved as a beneficial alternative approach to cure X-SCID patients carrying mutations of γc, a malignant lymphoproliferation occurred in 5 out of 20 patients enrolled into the two different trials (20, 58), not observed in gene therapy trials for SCID due to ADA deficiency (59). To explain these adverse events, studies were conducted to define whether the retroviral insertional mutagenesis could have played a role. In 4 cases an aberrant transcription and expression of *LMO2* was clearly documented (60). Even though the other patients may have the vector integration near LMO2 or other oncogenes (60), it is also conceivable that the transgene could have a role *per se* in cell cycle progression. Of note, development of leukemia, similar to other cancers, requires multiple genetic changes caused by a diverse group of genes that inhibit apoptosis and/or provide growth advantage to the leukemic cells (61). 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 (62). 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 (63). These results suggest that insertional mutagenesis may not be the only cause of laeukemogenesis and that the expression levels of γc could influence the cell cycle progression directly or its effect being mediated by cytokines triggers.

It has been documented that γc receptors activity enhances leukemogenesis (64). 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 nontargeting 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, which allowed us to demonstrate 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 (64). 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 (65). 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 (66, 67).

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 (57, 68-71) and STAT5 is considered a transforming agent in lymphoma and other cell types (72), therefore we found that the reduction of γc amount also inhibits STAT5 activation and its subsequent nuclear translocation, which follows GHR 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 (73). Moreover, a putative role of GH as a cofactor in tumor growth is plausible, since several carcinomas express GHR (74). In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events (75).

In conclusion, our 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. Therefore, since

results of gene therapy trials for X-SCID have been very promising, to achieve safer results, the modulation of the transgene expression could help reduce the risk of undesirable events.

These data have been published as Article on *The Journal of Immunology*, for the manuscript see below.

The Cellular Amount of the Common γ-Chain Influences Spontaneous or Induced Cell Proliferation[1]

Stefania Amorosi,* Ilaria Russo,* Giada Amodio,* Corrado Garbi,† Laura Vitiello,†‡ Loredana Palamaro,* Marsilio Adriani,* Ilaria Vigliano,* and Claudio Pignata[2]*

Mutations of the *IL2RG* encoding the common γ-chain (γ_c) lead to the X-linked SCID disease. Gene correction through ex vivo retroviral transduction restored the immunological impairment in the most of treated patients, although lymphoproliferative events occurred in five of them. Even though in two cases it was clearly documented an insertional mutagenesis in *LMO2*, it is conceivable that γ_c could have a role per se in malignant lymphoproliferation. The γ_c is a shared cytokine receptor subunit, involved also in growth hormone (GH) receptor signaling. Through short interfering RNA or using X-linked SCID B lymphoblastoid cell lines lacking γ_c, we demonstrate that self-sufficient growth was strongly dependent on γ_c expression. Furthermore, a correlation between γ_c amount and the extent of constitutive activation of JAK3 was found. The reduction of γ_c protein expression also reduced GH-induced proliferation and STAT5 nuclear translocation in B lymphoblastoid cell lines. Hence, our data demonstrate that γ_c plays a remarkable role in either spontaneous or GH-induced cell cycle progression depending on the amount of protein expression, suggesting a potential role as enhancing cofactor in lymphoproliferation. ***The Journal of Immunology*, 2009, 182: 3304–3309.**

Mutations of the *IL2RG* gene encoding the cytokine receptor common γ-chain (γ_c)[3] lead to the X-linked SCID (X-SCID) disease (1, 2). The severity of this disease makes it a medical emergency, which without any treatment leads to death in the first months of life. Bone marrow transplantation represents in this context the conventional therapeutic strategy for this form of immunodeficiency. This therapeutic approach confers to children affected by SCID at least a 70% chance of cure in the presence of a fully HLA-matched donor. Unfortunately, a fully compatible donor is not always available, thus limiting the successful use of this therapy. Moreover, the use of a not fully HLA-matched donor increases the immunologic complications such as graft-vs-host disease associated with a potential long-term decline in immune cell functions. These difficulties encouraged gene therapy trials (3). This strategy using ex vivo retroviral vectors has been proven as a corrective therapeutic approach for X-SCID in humans (4–9). Immunological reconstitution has been documented in 17 of 20 patients enrolled in two distinct clinical studies (3, 7). Unfortunately, five of these patients developed a lymphoproliferative disorder (10–12), not observed in gene therapy trials for SCID due to adenosine deaminase deficiency (13). This event was attributed to up-regulated expression of the *LMO2* oncogene, as a consequence of insertional mutagenesis (14). However, this event was clearly documented only in two cases. Even though the other patients may have the vector integration near *LMO2* or other oncogenes (14), it is also conceivable that the transgene could have a role per se in cell cycle progression. In keeping with this hypothesis, overexpression of γ_c transduced through a lentiviral vector into stem cells in a 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 (15). 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 γ_c control of cell cycle (16). These results suggest that insertional mutagenesis may not be the only cause of leukemogenesis and that the expression level of *IL2RG* could influence the cell cycle progression directly or its effect being mediated by cytokines triggers.

The γ_c is a transducing element shared among several IL receptors, whose activity was documented to enhance leukemogenesis (17), and is part of the intermediate- and high-affinity receptor of IL-2, that is essential for ligand internalization (18). In turn, this subunit activates several key signaling molecules such as JAK3, in which constitutive activation is frequently associated to autonomous cell growth and malignant transformation of lymphoid cells (19, 20). Recently, we demonstrated that γ_c subunit is also involved in growth hormone (GH) receptor (GHR) signaling in B lymphoblastoid cell lines (BCLs) (21). GH in BCLs obtained from X-SCID patients was unable to induce cell proliferation and STAT5 activation (22). *IL2RG* gene transduction of X-SCID BCLs promptly restored these functional and biochemical events, eventually resulting in STAT5 nuclear translocation (21).

In this study, we show through γ_c silencing experiments that the molecule is actively involved in a concentration dependent manner in self-sufficient growth and GH-induced cell cycle progression of BCLs, its activation being mediated by STAT5 phosphorylation and nuclear translocation.

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[3] Abbreviations used in this paper: γ_c, common γ-chain; GH, growth hormone; GHR, GH receptor; BCL, B lymphoblastoid cell line; siRNA, small interfering RNA; X-SCID, X-linked SCID.

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Materials and Methods

Reagents

Recombinant human GH was obtained from Serono. The ECL kit was purchased from Amersham Biosciences. The Abs anti-γ_c, anti-JAK3, anti-β-actin, anti-histone 3 (H3), anti-phosphotyrosine, anti-STAT5 were purchased from Santa Cruz Biotechnology. The neutralizing anti-IL-2 and anti-IL-4R mAbs were purchased from R&D Systems. Acrylamide and bisacrylamide were obtained from Invitrogen. Prestained molecular mass standards were obtained from Bio-Rad. The small interfering RNA (siRNA) duplexes specific for γ_c and the control nontargeting siRNA were obtained from Invitrogen. The control nontargeting pool contains nontargeting siRNAs with guanine cytosine content comparable to that of the functional siRNA but lacking specificity for known gene targets. Except where noted, other reagents were from Sigma-Aldrich.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from four X-SCID patients and six normal donors of heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation (21). BCLs were generated by EBV immortalization of patients and control PBMC using standard procedures. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% FBS (Invitrogen), 2 mM/L L-glutamine (Invitrogen), and 50 μg/ml gentamicin (Invitrogen), and cultured at 37°C, 5% CO_2. In self-sufficient growth experiments, BCLs were cultured in DMEM/F12 without FBS and supplemented with 2 mM/L L-glutamine.

In neutralization experiments, BCLs were cultured in 96-well plates, preincubated with the neutralizing mAbs 202 or 230 at the indicated concentrations.

siRNA transfection

Preparation of the cells before Lipofectamine 2000 transfection was performed according to the manufacturer's recommendations. Briefly, for each transfection 1×10^6 BCLs in 1 ml were treated with 20 μl of 50 μM siRNAs specific for the γ_c or equal amount of the control nontargeting siRNA. The siRNAs were solubilized and formed complexes separately with the lipid-based transfectant, Lipofectamine 2000. The siRNA-lipofectamine complexes were transfected into the cultured cells in a 24-well plate and incubated for the time indicated in the text. Throughout the experiments, cell vitality was monitored continuously by trypan blue exclusion assay. Furthermore, 96 h after the transfection, the cells were washed, placed in fresh culture medium and used for further analysis, as described.

CFSE labeling

Cell proliferation was measured by the cell surface stain CFSE. BCLs (1×10^6) were labeled with 1.7 μM CFSE in PBS just before culturing for the indicated times using a serum-free medium. After 2 min at room temperature, BCLs were washed in FBS and PBS and cell division accompanied by CFSE dilution was analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

[^{3}H]thymidine incorporation assay

Cell number was assessed by counting cells after trypan blue dye exclusion staining. BCLs were cultured for different time ranging between 6 h and 4 days at a density of 1×10^5 viable cells/200 μl well in triplicate wells (96-well microtiter plates, Falcon; BD Biosciences). Cultures were pulsed with 0.5 μCi [^{3}H]thymidine for 8 h (or 6 h in the short-term cultures) before harvesting and the incorporated radioactivity measured by scintillation counting. Where indicated (see Fig. 5), recombinant GH was added to the culture at 50 ng/ml. The results are expressed as mean cpm for triplicate cultures.

Immunoprecipitations and Western blotting

Following transfection and appropriate recombinant GH stimulation, BCLs were lysed in 100 μl of lysis solution (20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) on ice for 45 min. Protein concentration was determined by Bio-Rad protein assay. The cell lysates were stored at −80°C for Western blot analysis. Nuclear extracts were prepared by the method previously described (21). Proteins were electrophoretically separated on 10% Tris glycine SDS-PAGE gels. Proteins were transferred onto nitrocellulose transfer membranes (Schleicher & Schuell). Membranes were incubated with the specific primary Abs. Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked Abs. ECL detection system was used for visualization.

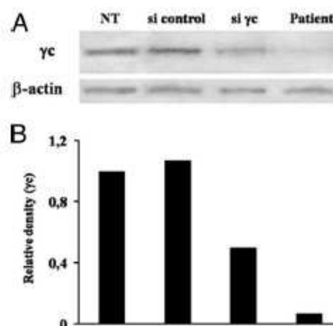


FIGURE 1. The γ_c silencing by siRNA induced a reduction in protein amount. *A*, After 96 h of culture, control BCLs transfected with nontargeting siRNA (si control), γ_c siRNA (si γ_c), or nontransfected (NT), and X-SCID BCLs (patient) were lysed and γ_c total amount was measured by Western blotting. Membranes were incubated as indicated with Abs anti-γ_c and anti-β-actin, used as loading control. *B*, Densitometric analysis of the above Western blot. ImageJ program was used to generate the data.

Equal loading was confirmed after stripping and reprobing with anti-β-actin or anti-histone 3 Abs.

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 μg/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 Ab for phosphotyrosine.

Densitometric analysis was performed on a Windows personal computer, using the public domain Java image processing program ImageJ (developed at the National Institutes of Health and at (http://rsb.info.nih.gov/ij/index.html). Each signal has been evaluated in comparison with the control lane 1 and equalized for the loading control, applying the following formula: (sample lane/control lane)/loading control ratio.

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 (21). 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). At least 100 cells per condition were analyzed in each experiment to determine the rate of STAT5 nuclear translocation.

Results

Common γ_c silencing inhibits self-sufficient growth and down-regulates constitutively activated JAK3 in B cell lines

To define an intrinsic mitogenic property of γ_c dependent on the amount of the protein, we used in vitro cellular models containing different amounts of γ_c. In particular, BCLs from normal subjects, cells transduced with lipid vector containing nontargeting siRNA, BCLs transduced with siRNA to knockdown γ_c expression and BCLs from X-SCID patients were used. The transfection efficiency was tested using fluorescent oligonucleotides under fluorescent microscope. Levels of γ_c were evaluated by Western blotting of whole cell lysates. The γ_c expression was reduced to 50% of the control in γ_c-silenced BCLs and completely undetectable in X-SCID BCLs (Fig. 1*A*). Densitometric analysis is shown in the histogram in Fig. 1*B*.

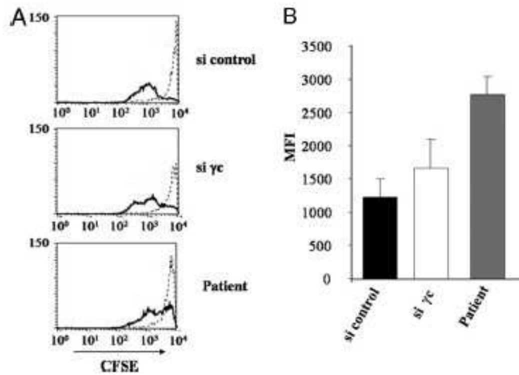


FIGURE 2. The γ_c was involved in self-sufficient growth of BCLs. *A*, After 12 h of starvation, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested and cell proliferation was assessed using cytofluorimetry for CFSE intensity. CFSE dilution profiles are shown. Histograms show on gated cells the number of events (y-axis) and the fluorescence intensity (*x*-axis) 6 h following the start of the culture. Dashed lines represent the start of the culture. *B*, Mean fluorescence intensity (MFI) of gated CFSE-positive cells maintained in the same conditions as described in *A*.

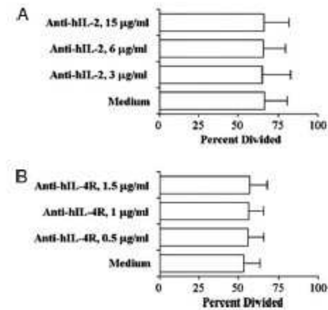


FIGURE 4. The γ_c-activating cytokines did not affect self-sufficient growth of BCLs. *A*, Control BCLs were treated with the indicated concentration of Anti-human IL-2, were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested and cell proliferation was assessed using cytofluorimetry for CFSE intensity. *B*, Control BCLs were treated with the indicated concentration of anti-human IL-4R and analyzed as described in *A*. The percentage of cells that divided is shown. Error bar indicates 1 SD.

Because self sufficiency in growth has been suggested as one of the six acquired capabilities of cancer phenotype (23), we examined the abilities of these previously mentioned BCLs to grow in serum-deficient conditions, upon trypan blue exclusion assay. We labeled BCLs, after 12 h of starvation, with CFSE, a dye that allows proliferative history to be visualized, and assessed the CFSE dilution profile at different time points ranging between 6 h and 7 days to establish the rate of spontaneous cell proliferation (data not shown). Informative data on differences between previously described BCLs were appreciable as soon as 6 h from the start of the culture, presumably because of the high proliferation rate of BCLs as compared with normal mononuclear cells. At this time, only 14% of control cells retained the dye, indicating a high proliferation rate, compared with 26% of γ_c-silenced cells and to 50% of X-SCID BCLs (Fig. 2*A*). In addition, the final mean fluorescence intensity, reflecting CFSE-derived fluorescence per cell, were 1378 units in control, 1825 in γ_c-silenced, and 2866 in X-SCID, thus confirming that only in control cells a substantial dye dilution occurred (Fig. 2*B*).

We then cultured viable cells for 4 days in a serum free medium and 0.5 μCi of [^{3}H]thymidine were added 8 h before harvesting. γ_c-silencing reduced cell proliferation of unstimulated BCLs by 69% as compared with control cells. In X-SCID BCLs the extent of the reduction was higher corresponding to 97% of control BCLs (Fig. 3*A*). These data were, therefore, in keeping with the results of CFSE experiments. Moreover, to prove that the effect observed in the CFSE experiments in the 6 h cultures were really indicative of cell proliferation, several time-course experiments with both techniques were performed at the beginning of the study. These data indicate that the proliferation rate of these cells is comparable using the two methods in the first 12 h (see supplemental materials S1 and S2),[4] indicating that the CFSE dilution reflects a real cell division. Furthermore, the addition of mitomycin prevents staining dilution, providing further evidence that CFSE signals reflect a real cell division. In particular, after 6 h of culture 12, 31, and 59% of the control, silenced or SCID patient cells, respectively, retained the dye as compared with the 100% of stained cells at the beginning of the culture (see supplemental material S3).[4]

Because JAK3 is essential for autonomous proliferation being physically linked to γ_c, we further investigated the role of γ_c in self-sufficient growth, evaluating JAK3 activation. Of note, JAK3 proteins are constitutively phosphorylated in EBV-immortalized B cells and other malignant cells (24). Thus, we evaluated the effect of different amount of γ_c on the levels of constitutively phosphorylated JAK3 protein (phospho-JAK3). After 12 h of serum-free

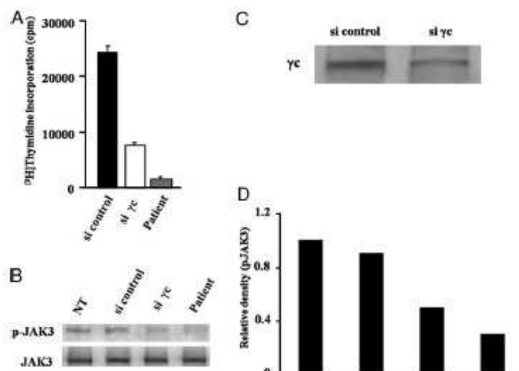


FIGURE 3. The γ_c protein depletion had effect on spontaneous cell proliferation and on activated JAK3 levels. *A*, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were tested for their ability to proliferate in serum-free medium. Cultures were maintained in serum-free medium for 4 days and pulsed with [^{3}H]thymidine for the final 8 h. Radioactive incorporation was counted. Error bar indicates 1 SD. *B*, Unstimulated BCLs, after 12 h of starvation, were immunoprecipitated with anti-JAK3 Ab and tested in Western blot with anti-phosphotyrosine mAb. Equivalent loading was controlled by reprobing the membrane with JAK3 Ab. Nontransfected (NT) BCLs were also tested. *C*, Control BCLs, transfected with nontargeting siRNA (si control) and γ_c siRNA (si γc), were lysed and γ_c total amount was measured by Western blotting. *D*, Densitometric analysis of Western blot shown in *B*.

[4] The online version of this article contains supplemental material.

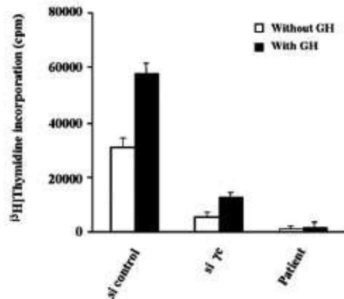


FIGURE 5. Silencing of γ_c inhibited GH-induced proliferation. Cell proliferation of BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) or nontransfected (NT) stimulated with recombinant GH (50 ng/ml) was evaluated through [^{3}H]thymidine incorporation assay. Error bar indicates 1 SD.

culture, whole cell lysates were immunoprecipitated with anti-JAK3 Ab and the obtained membranes were immunoblotted with anti-phosphotyrosine mAb. A higher constitutive activation of JAK3 was found in control BCLs, whereas a decrease in phospho-JAK3 levels was observed in γ_c-silenced and in X-SCID BCLs, despite a comparable amount of the whole protein (Fig. 3*B*). The amount of pJAK3 paralleled the amount of γ_c, shown in Fig. 3*C*. The densitometric analysis of phospho-JAK3 equalized for total JAK3 is shown in a histogram in Fig. 3*D*.

Evidence is available that γ_c-dependent cytokines, as IL-2 and IL-4, may be secreted in EBV-infected B cells (25–27). Thus, to define whether the mitogenic effect of γ_c was independent or dependent from receptor engagement of these endogenous γ_c-activating cytokines, we used neutralizing mAbs anti-IL-2 or anti-IL-4R in the CFSE-based proliferative assay. As shown in the Fig. 4, the neutralizing mAbs did not reduce at any concentration spontaneous cell proliferation.

The γ_c silencing inhibits GH-induced cell proliferation and subsequent STAT5 activation

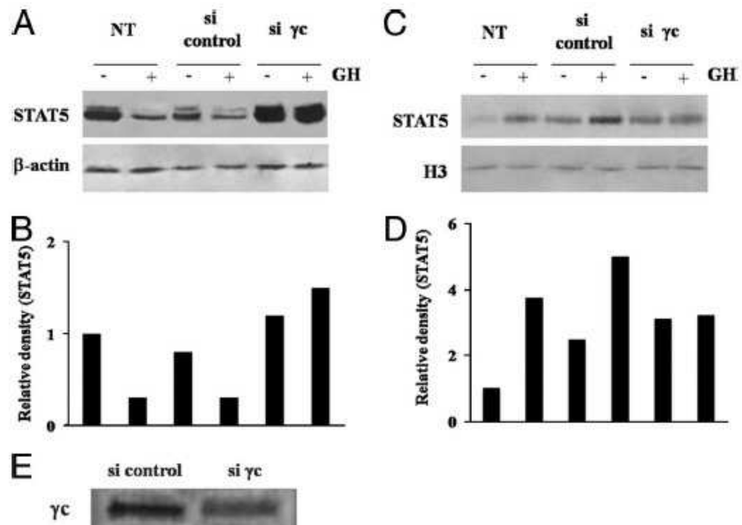
It has been reported that GH enhances BCLs proliferation in vitro (28) and that γ_c is functionally linked to GHR (21). Moreover, it has been described the association between lymphoproliferative events and supraphysiological doses of GH, both in mice and humans (29).

Because growth factors may participate in autocrine or paracrine loops that affect tumor cells growth or survival and autocrine production of GH is able to induce cellular transformation (30), we evaluated the response to GH stimulation of X-SCID BCLs, control cells and γ_c-silenced BCLs to assess whether γ_c amount could influence GH response. Recombinant GH at a concentration of 50 ng/ml enhanced proliferation of control BCLs. In γ_c-silenced or X-SCID BCLs, recombinant GH induced proliferation at a much lower extent, corresponding to 28% and 5% of the control, respectively (Fig. 5).

Because the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (31–35) and STAT5 is considered a transforming agent in lymphoma and other cell types (36), we then evaluated whether γ_c-silencing had effect on GH-induced STAT5 subcellular localization. Nuclear and cytoplasmic extracts from BCLs, unstimulated or treated with 500 ng/ml recombinant GH, were evaluated by immunoblot for the overall amount of STAT5. Recombinant GH induced a rapid decrease of the cytoplasmic amount of STAT5 in control BCLs and in BCLs treated with control nontargeting siRNA, differently from γ_c-silenced BCLs, in which no effect on the protein amount was observed (Fig. 6*A*). This finding was inversely correlated with the amount of the nuclear form of the molecule. In fact, in control BCLs and in BCLs treated with control siRNA, an increase of nuclear STAT5 amount was observed after recombinant GH stimulation, differently from what observed in γ_c-silenced BCLs, in which no change was observed (Fig. 6*C*). These data, representative of different experiments, reflect a real subcellular redistribution of the molecule in that no difference in the cytoplasmic β-actin and nuclear histone H3 expression was observed. The densitometric analysis normalized for the housekeeping molecules is shown in Fig. 6, *B* and *D*.

Furthermore, we looked at STAT5 subcellular localization using confocal microscopy. In control unstimulated BCLs, only 10% of cells showed a nuclear localization of STAT5, being the protein mainly concentrated in the cytoplasm. GHR perturbation through recombinant GH stimulation at a concentration of 500 ng/ml

FIGURE 6. Silencing of γ_c influenced STAT5 nuclear translocation in B cell lines. Cells from BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were analyzed for subcellular localization of STAT5. Cells were stimulated with 500 ng/ml recombinant GH for 30 min. *A*, Cytoplasmic amount of STAT5. Equivalent loading was controlled by reprobing the membrane with β-actin. *C*, Nuclear fraction of STAT5. Equivalent loading was controlled by reprobing the membrane with histone H3. *B* and *D*, Densitometric analysis of the Western blots from BCLs in *A* and *C*. *E*, Control BCLs, transfected with nontargeting siRNA (si control) and γ_c siRNA (si γc), were lysed and γ_c total amount was measured by Western blotting.



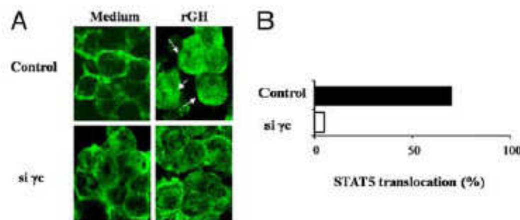


FIGURE 7. The γ_c silencing impairs recombinant GH-induced STAT5 subcellular redistribution. *A*, Evaluation of STAT5 subcellular localization through confocal microscopy. Control or γ_c-silenced BCLs (si γc) were cultured in the absence or presence of 500 ng/ml recombinant GH for 30 min. Arrows indicate exemplificative cells with nuclear STAT5 staining. Nucleoli are not stained. *B*, The percentage of STAT5 nuclear translocation is shown. These data represent an analysis of independent observations.

induced STAT5 nuclear localization in the 70% of cells. Differently, recombinant GH stimulation of γ_c-silenced BCLs had negligible effects on nuclear STAT5 migration, resulting in a 5% increase of positively stained cells as compared with unstimulated cells (Fig. 7).

Discussion

Our results indicate that silencing of γ_c induces a substantial decrease of protein amount in BCLs, that allowed us to demonstrate a direct involvement of γ_c in self-sufficient growth of BCLs in a concentration dependent manner. Moreover, we documented that the amount of γ_c also influences the response of BCLs to GH-induced proliferation and STAT5 subcellular redistribution that follows GHR perturbation. These data add new evidence for a possible intrinsic mitogenic role of γ_c related to its cellular amount. This biologic effect could be either direct, thus related to the molecule per se, or indirect and mediated by the participation to cytokine-receptors signaling.

The intrinsic property of γ_c in cell cycle progression has been long debated. In fact, although gene therapy trials have been proved as a beneficial alternative approach to cure X-SCID patients carrying mutations of γ_c, a malignant lymphoproliferation occurred in 5 of 20 patients enrolled into the trials, alarming the scientific community (3). To explain these adverse events, studies were conducted to define whether the retroviral insertional mutagenesis could have played a role. In two cases, an aberrant transcription and expression of *LMO2* was clearly documented (14). However, for the remaining patients there isn't any evident demonstration of *LMO2* alteration due to random insertions that could be causative in transformation. An in vivo expansion of cell clones has also been documented in other gene therapy trials. Two patients treated with gene therapy for X-linked chronic granulomatous disease developed myeloid proliferation. Of note, in these cases cell clones didn't exhibit any self-renewal capacity. This observation would imply that there is no evidence of continued abnormal growth of clones containing insertionally activated growth-promoting genes (37). Of note, development of leukemia, similar to other cancers, requires multiple genetic changes caused by a diverse group of genes that inhibit apoptosis or provide growth advantage to the leukemic cells (38). In this study, we demonstrate that γ_c exerts a role in cell cycle progression in a strictly concentration dependent manner. We, also, found that the amount of constitutively activated JAK3 parallels the extent of γ_c expression. This finding is intriguing, in that constitutively active or hyperactive JAK proteins have crucial roles in hematopoietic malignancies, by promoting oncogenic transformation and uncontrolled blood cell production (17). 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 (20). 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 (39, 40). Moreover, the role of JAK3 in cell destiny is emphasized by the finding that JAK3 mutations cause a SCID phenotype, thus implying its role in lymphoid development (41). JAK3 has also the capacity to activate DNA synthesis and protooncogenes, such as c-*myc* and c-*fos* (42).

In this study, we also observed that the participation of γ_c in GHR signaling apparatus and, in particular, in GH-induced STAT5 activation and nuclear translocation was also dependent on the extent of its molecular expression. Thus, the concentration-dependent mitogenic effect of γ_c could be favored by the participation of γc in GHR signaling. Of note, it should be mentioned that experimental studies document a role for GH in the initiation or promotion of tumorigenesis, raising the possibility that patients treated with GH might be at increased risk of cancer (29). Moreover, a putative role of GH as a cofactor in tumor growth is plausible because several carcinomas express GHR (43). In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events (44).

Our data would imply that the expression levels of γ_c in hematopoietic cells are crucial for the maintenance of cell growth control. Whether our data may have direct implications in the understanding of the pathogenesis of the lymphoproliferative events occurring during gene therapy trials for X-SCID remains to be clarified. Even though, under ordinary conditions, γ_c is expressed at a normal extent in cells, transduced with retroviral vectors containing wild-type γ_c, our data indicate that altering the expression levels of the protein could be important in modifying cell cycle control mechanisms. Our findings are in keeping with a recent study, which demonstrates that in X-SCID murine model, T cell lymphomas and thymic hyperplasia occur in a third of the cases treated with lentiviral vectors containing wild-type γ_c (15). This event was independent from common integration sites and, thus, not attributable to insertional mutagenesis, but rather to an intrinsic oncogenic property of the transgene and, presumably, to the overexpression of the molecule (15). Furthermore, by searching in Mouse Retroviral Tagged Cancer Gene Database, integration in *Il2rg* has been found in two cases of retrovirally induced leukemias (45).

In conclusion, our data demonstrate a direct relationship between the amount of γ_c expression and its role in cell cycle progression. Therefore, because results of gene therapy trials for X-SCID have been very promising, to achieve safer results, the modulation of the transgene expression could help reduce the risk of undesirable events.

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Disclosures

The authors have no financial conflict of interest.

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§1.3 Role of γc on spontaneous cell cycle progression in malignant cell lines

Cell cycle progression is a highly organized and regulated process that controls cell proliferation (76). Cytokines that signal through receptors sharing the γc lead to transition into the cell cycle and thus proliferation (77). The entry of cells into the cell cycle is controlled by an ordered expression/activation of cyclins (78). IL-2R through γc appears to activate a variety of downstream signaling pathways that converge on the regulation of Bcl-2 (79), including PI3K and MAPK activation (29) and transcription of the *c-myc* gene (79). In turn, c-myc cooperates with STAT5 to induce the expression of cyclin D and to promote proliferation (80-82). It is clear that alterations in Bcl-2 family members levels exert potent effects on cellular survival and, namely, Bcl-2 overexpression can be tumorigenic (83). Moreover, γc is required for a wide range of signaling inputs that induce cell proliferation through cyclin D3 expression (84).

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 (**Figure 4A**). Programmed cell death is mainly mediated by activation of several caspases (85). These molecules exist as pro-forms that are activated by cleavage by the upstream caspase in the cascade (85). 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 (**Figure 4B**). Caspase-independent cell death has been attributed to mitochondrial damage (86), which can be regulated by Bcl-2 family members (87, 88). 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 (89) and Bcl-XL (90), 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 (**Figure 4B**). 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 (**Figure 4C**). However, evidence exists that autophagy can plays an active role in cell death, by contributing to cell death in unfavourable settings such as nutrient or growth factors deprivation (91). In keeping with this, probably γc could have a role in the autophagy process.

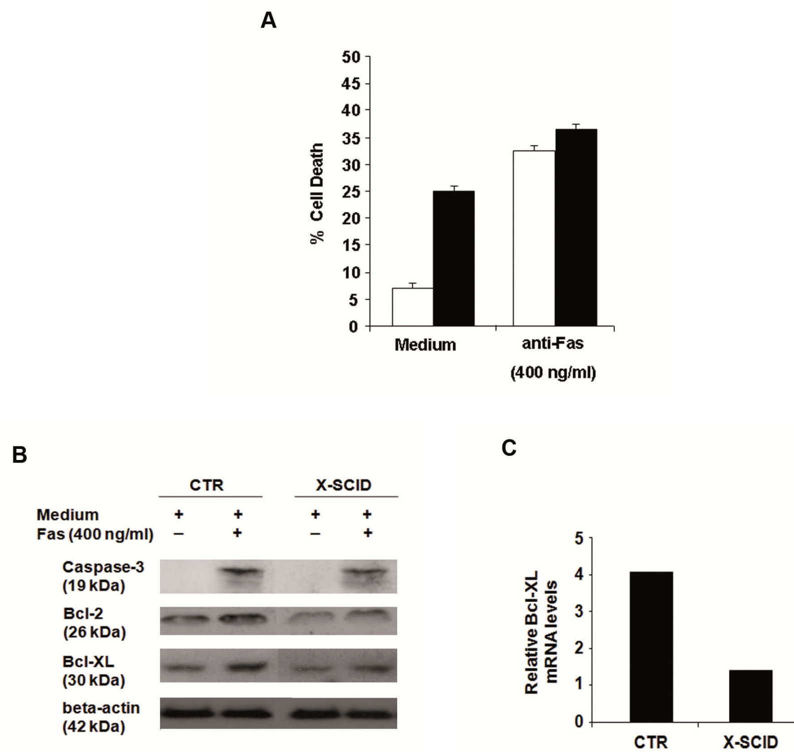

Figure 4. Deficiency in the expression of γc has effect on cell survival. (A) BCLs were cultured in the absence or presence of 400 ng/ml anti-Fas for 6 hours. The percentage of cell death was evaluated through trypan blue staining. Filled bars indicate BCLs from X-SCID patients; open bars BCLs from healthy donors (CTR). Data are expressed as mean (± SD) of 6 experiments. (B) BCLs were either cultured in medium alone or stimulated with anti-Fas. After 6 hours, 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.

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 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 through quantitative real-time PCR. IL-2Rγ mRNA levels was greatly increased in leukemic cells as compared with the control (**Figure 5**).

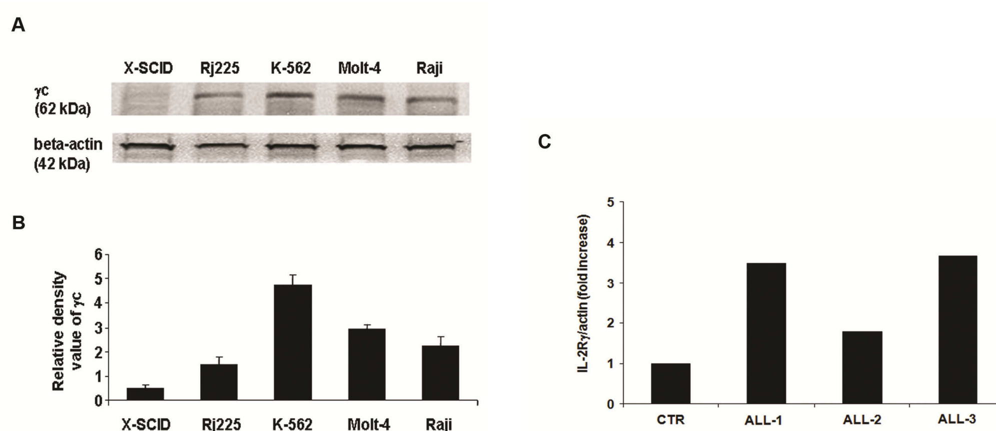

Figure 5. Different γc expression levels in malignant cell lines. (A) X-SCID BCLs, Burkitt lymphoma cell line (Raji), the chronic myelogenous leukemia cell line (K-562), the human T-acute lymphoblastic leukemia cell line (Molt-4) and Raji isogenic derivative (Rj225) were lysed and immunoblotted for γc 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 6 distinct experiments. (C) Primary leukemic cell lines, consisting of acute lymphoblastic leukemia (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.

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 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. No proliferation was observed in X-SCID BCLs (**Figure 6A-C**). Moreover, the proliferation of these

cell lines was also assessed by ^{3}H-thymidine incorporation assay. The 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, $p < 0.05$) (**Figure 6D**).

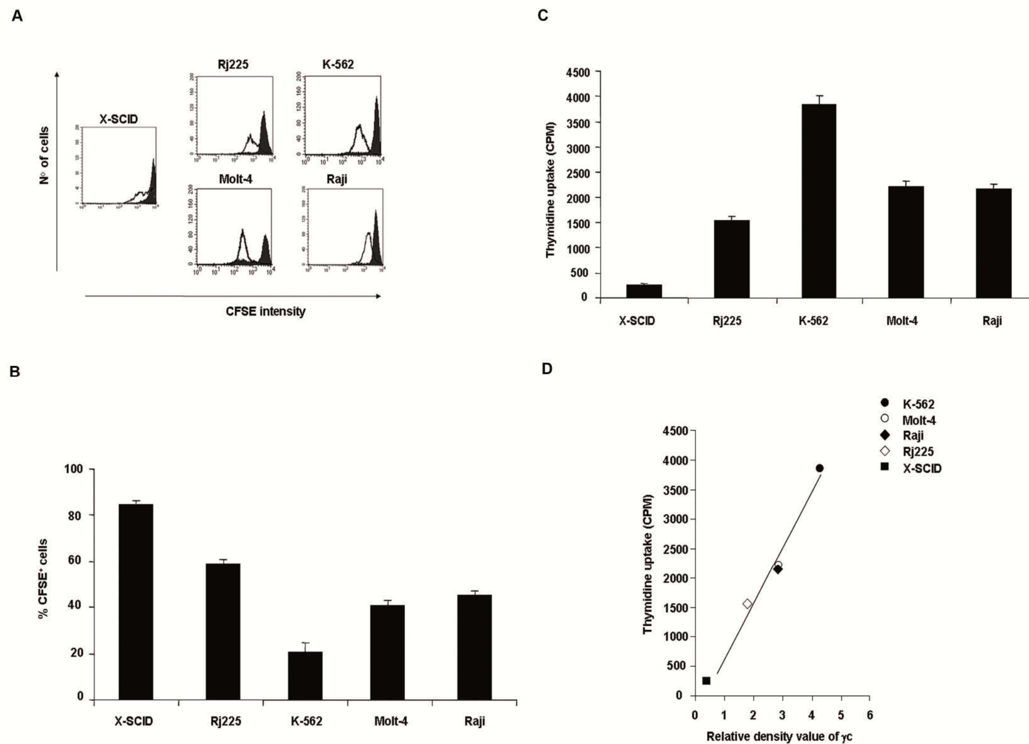

Figure 6. Relationship between γc expression and spontaneous cell growth in malignant cell lines. (A) X-SCID BCLs, Raji, K-562, Molt-4 and Rj225 were cultured in the absence of serum and stained with 1.7 μM CFSE. After 6 hours of culture, cells were analyzed by flow cytometry. Histograms show CFSE profiles 6 hours 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 3 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 hours. Data represent mean (± SD) of 6 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, $p < 0.05$).

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. As shown in Figure 4A-B, 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 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 approximately 85%. In X-SCID cells, IL-2Rγ mRNA was undetectable (**Figure 7A-C**). Moreover, 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 (**Figure 7D**). Taken together, these data confirm that γc plays a key role in the proliferation of these malignant cell lines.

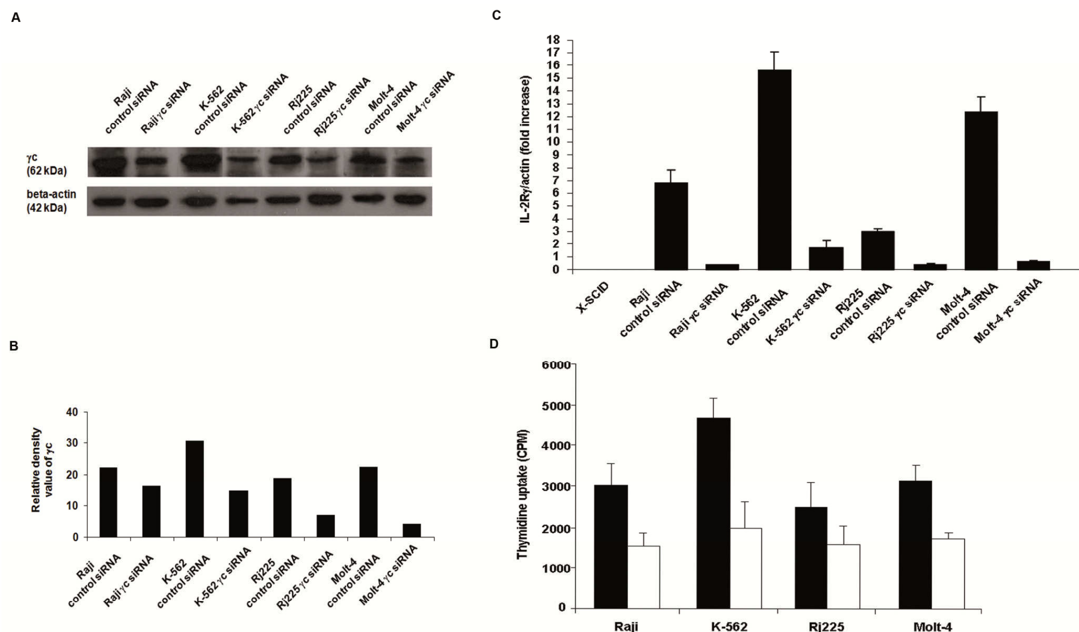

Figure 7. Effect of γc siRNA transfection on the expression level of protein and cell proliferation of malignant cell lines. (A) Western blot analysis of γc and beta-actin protein expression in Rj225, K-562, Molt-4 and Raji transfected with γc siRNA or transfected with negative control siRNA after 96 hours 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 5 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 5 distinct experiments.

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 (92). 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 (78). 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 (93). Namely, cyclins A2 and B1 have been implicated in the pathogenesis of cancer and are overexpressed in several tumors (94, 95). Evidence indicates that these cyclins are key components of the cell-cycle machinery (96) and, in particular, cyclin A is expressed at high levels in hematopoietic stem cells and is essential for their proliferation (97). In our study, we observed that the expression of A2 and B1 cyclins strongly paralleled the proliferative capability of malignant cell lines (**Figure 8B**). Interestingly, a positive correlation between the amount of γc and the expression of cyclins A2 and B1 was also found (**Figure 8C, D**). 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 (**Figure 8A**).

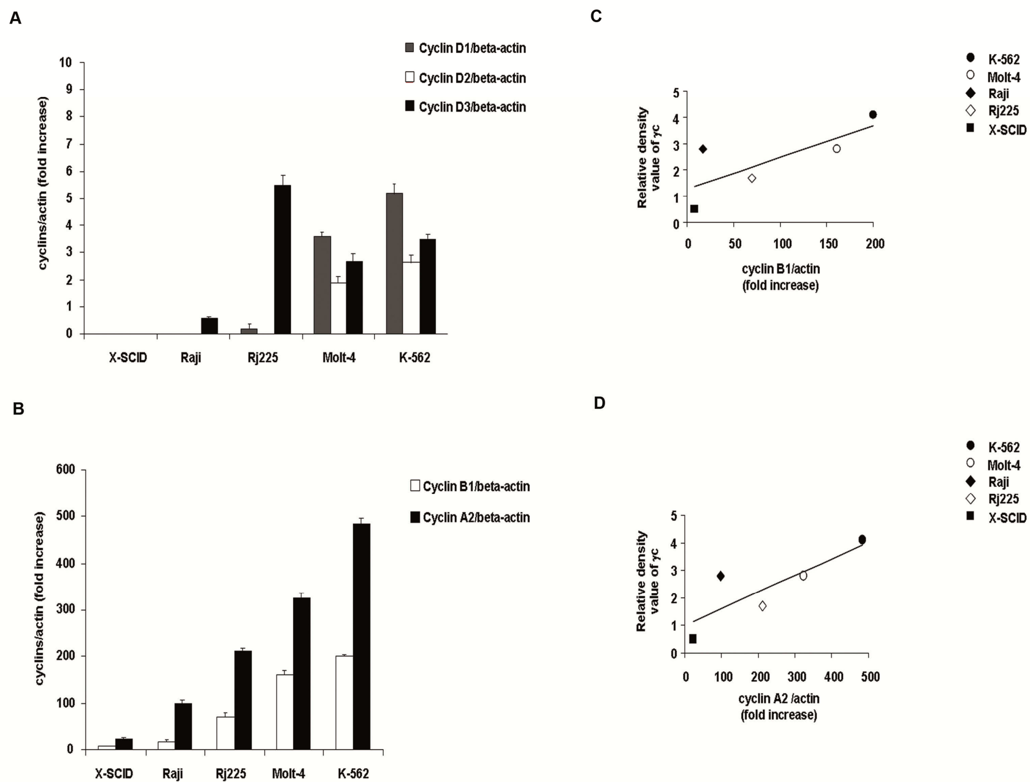

Figure 8. Cyclins expression is upregulated in malignant cell lines. (A-B) RNAs extracted and reverse-transcribed were analyzed for the expression of D1, D2, D3 and A2, B1 cyclins by qRT-PCR. Histograms show the relative gene expression as cyclin/actin fold increase. Relative expression of cyclins were 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.

D-type cyclins are strongly expressed in many malignancies. Overexpression of cyclin D1 protein was documented in many forms of cancer, including breast cancer (98), while overexpression of cyclin D2 was noted in a wide range of B cell malignancies, such as B cell chronic lymphocytic leukemia (99). 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 (100). However, while A and B type cyclins seem to be vital and necessary components of cell cycle progression (97), 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 (101). 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 (101). Anyway, a critical role for oncogenic transformation of D-type cyclins is a well established feature.

§1.4 Conclusive remarks

It's noteworthy that 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 (102). There is evidence documenting that immune cells express GH-R (42). It has also recently been shown a novel dependence of GH signaling on the γc cytokines receptor in certain cell types, suggesting the interplay between endocrine and immune system (42). GH-R can promote cell cycle progression of lymphoid cells and of a wide variety of other cells. Indeed, we documented a direct involvement of γc in self-sufficient growth and GH induced proliferation in a concentration dependent manner of the molecule (103). GH-R signaling apparatus also involves potent mitogenic molecules such as STATs that play a role in cell proliferation (104). Moreover, it was previously reported that *IL-2RG* cooperates with *LMO2* in inducing hematopoietic tumors by studies of insertional mutagenesis in mice (105), thus giving a potential explanation to lymphoproliferative disorders occurring during gene therapy trials for X-SCID (106, 107). It is noteworthy that, differently from X-SCID, no clonal lymphoproliferation has been reported, to date, in patients receiving gene therapy for ADA deficiency (108), despite the observation of a similar frequency of integration sites near *LMO2* and other proto-oncogenes (109). Furthermore, a recent study, based on an experimental model of gene transfer in $\gamma c^{-/-}$ mice, documented that γc overexpression could exert oncogenic properties by itself (110).

Moreover, 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.

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.

CHAPTER II

“Nude/SCID syndrome due to alterations in FOXN1”

In humans, the thymus which is the only organ capable to support the differentiation and selection of mature T lymphocytes (111). The prenatal thymus development, the maintenance of a proper thymic microenviroment and the efficient T-cell production require an appropriate crass-talk between thymocytes and thymic stromal cells (112). The postnatal thymic involution results in dramatically reduced T-cell generation in an age-dependent manner (113). The thymic stromal compartment consists of several cell types that collectively enable the attraction, survival, expansion, migration and differentiation of T-cell precursors. The thymic epithelial cells (TECs) constitute the most abundant cell type of the thymic microenvironment and can be differentiated into morphologically, phenotypically and functionally separate subpopulations of the thymus (114). The Foxn1 gene is expressed in skin epithelial cells, hair follicles and TECs. Foxn1 function, as a transcriptional activator, is absolutely required for the normal differentiation of hair follicles and TEC (115). Foxn1 encodes a transcription factor whose function is essential for subsequent epithelial differentiation; without it, colonization of the anlage by thymocyte progenitors fails (116) and thymopoiesis is aborted, resulting in severe immunodeficiency (117). In fact, both mice and humans mutations in *Foxn1* gene resulting in a complete absence of the protein, display the ‘nude’ phenotype, which is characterized by hairlessness and congenital athymia.

§2.1 T-cell development and Thymus

The thymus is the primary lymphoid organ that supports T cell differentiation and repertoire selection (118, 119). 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. Following the entry into the thymus through the cortico-medullary 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 (**Figure 9**).

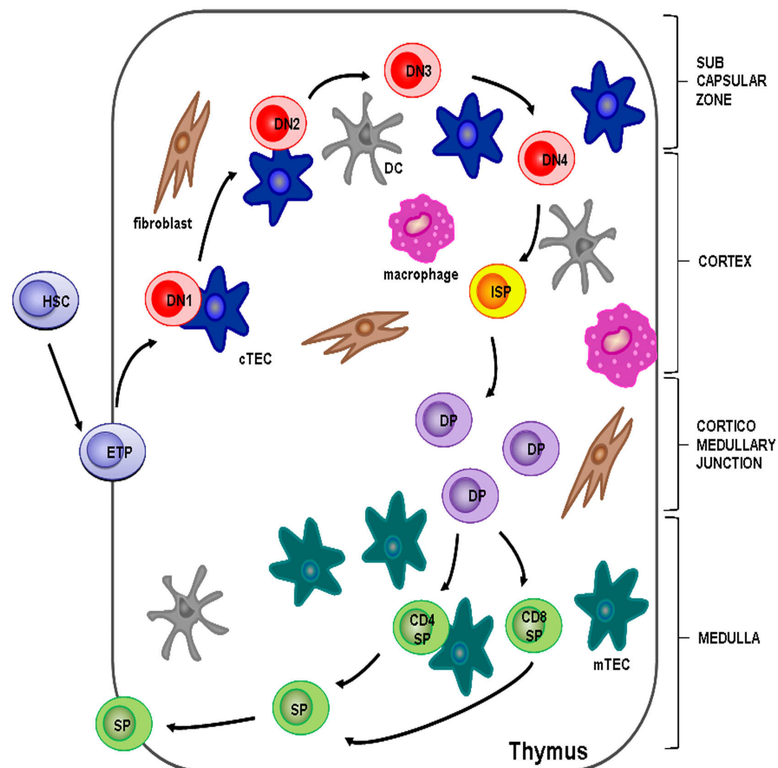

Figure 9. Steps of the T-cell development

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) (120). The immature thymocytes journey through the thymus has also the additional effect of promoting the differentiation of thymic stromal precursors into mature TECs, thus playing an important role in the formation of the thymic microenvironment (121-124). In particular, thymocytes during the DN1-DN3 stages participate to the differentiation process of TEC precursor cells into cortical TECs (cTECs). The DN2 to DN3 stage transition requires the expression of a different arrays of genes, 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. These cells become competent to undergo β-selection and express the pre-TCR complex on their surface and reach the DN3 stage (125). 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 and become DP cells. Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells (126). At this site, take place the positive selection, where 'productive' T cells react to foreign antigens, but not to self antigens (111). 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 that are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity (111). SP thymocytes egress from the thymus as recent thymic emigrants (RTE), 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 (127-129).

§2.2 The clinical spectrum of the Nude/SCID phenotype

In 1966, Flanagan identified a new mouse phenotype characterized by loss of the hair. This mouse showed an abnormal keratinization in hair fibers, with follicular

infundibulum unable to enter the epidermis (130). These affected mice also showed an inborn dysgenesis of the thymus (131) resulting in a compromised immune system lacking T cells (**Figure 10**). The mouse ʽnude' phenotype results from inactivating mutations in a single gene, originally named winged-helix-nude (*whn*)**,** recently known as forkhead box n1 (*foxn1*)(132).

Mice homozygous for the mutation ʽnude' are hairless have retarded growth, decreased fertility and die by 5 months of life for infections (130). The thymus is absent at birth (133) and there are very few lymphocytes in the thymus dependent areas of the spleen and lymph nodes (134). Since the abnormal, or even absent, thymus is the hallmark of the ʽnude' phenotype, these animals develop a profound T-cell deficiency and a severely impaired immune response of either cell-mediated and, indirectly, humoral immunity. ʽNude' 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 ʽnude' mice do not lack precursors of antibody forming cells. This indicated that antibody forming cells can mature in the absence of the thymus, although at a slower rate (135). In ʽnude' mice lymph nodes, the outer cortex with primary nodules and the medullary cords are normal. In the spleen sections from the ʽnude' 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 (134).

Figure 10. 'Nude' mouse phenotype

For many years the human counterpart of 'nude' mouse phenotype has been erroneously considered the DiGeorge syndrome that occurs spontaneously and is mainly characterized by thymic hypoplasia or aplasia. Children with DiGeorge syndrome also have lymphopenia, with a reduction of T cells that are poorly responsive to common mitogens (136).

The discovery of the human phenotype completely equivalent to the 'nude' mouse phenotype began with the identification of two sisters, whose clinical phenotype was characterized by congenital alopecia, eyebrows, eyelashes, nail dystrophy and several T-cell immunodeficiency (18). The two patients were born from consanguineous parents who originated from a small community of south of Italy that may be considered geographically and genetically isolated. This led to consider the syndrome as inherited as an autosomal recessive disorder. The T-cell defect was characterized by a severe functional impairment, as shown by the lack of proliferative response to common mitogens.

Due to the similarities between the human clinical features and the 'nude' mouse phenotype, a molecular analysis of the *FOXN1* gene was performed in these patients and revealed the presence of a C-to-T shift at 792 nucleotide position in the cDNA sequence. This mutation leads to a nonsense mutation R255X in exon 5 with a complete absence of a functional protein (19), similar to the previously described rat and mouse *Foxn1* mutations. In humans, *FOXN1* is located on chromosome 17 (in mice, chromosome 11).

Later, the identification of the haplotype for the *FOXN1* locus, by analysing 47 chromosomes carrying the mutation R255X, led to identify the single ancestral event that underlies the human Nude/SCID phenotype. As this form of SCID is severe due to the absence of the thymus and the blockage of T-cell development, a screening program for prenatal diagnosis in this population was conducted for the identification of fetuses carrying the mutation.

Interestingly, additional studies have also reported on anomalies of brain structures, suggesting of a potential role of FOXN1 in brain embryogenesis, as also suggested by its expression in epithelial cells of the developing choroids plexus in mice, 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 (137, 138).

A report on an athymic Nude/SCID fetus gave us the unique opportunity to gain further insights into the prenatal ontogeny of T lymphocytes in humans. Since under certain circumstances, such as immunodeficiencies (139), maternal cells may cross the placenta and engraft into the fetus *in utero*, all samples were analyzed after exclusion of maternal cell contamination, by evaluating 15 highly polymorphic autosomal short tandem repeat loci through multiplex-PCR (**Figure 11**).

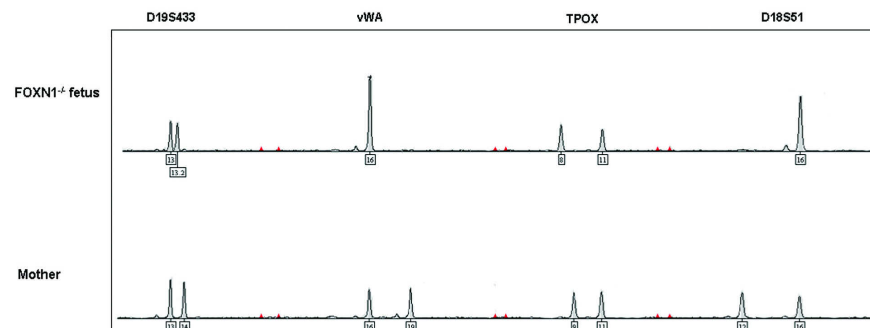

Figure 11. A representative electropherogram. Four short-tandem-repeat (D19S433, vWA, TPOX, D18S51) useful to exclude maternal contamination. Numbers denote allelic designations of individual loci.

The immunological and molecular events have been analyzed, with a particular attention to the expression of developmentally regulated markers of T-cell ontogeny, such as those ones expressed by RTE, and to the study of the TCR repertoire. Our results provide an evidence on the crucial role of FOXN1 in the early prenatal stages of T-cell ontogeny in humans, in that its alteration leads to a total blockage of $CD4^+$ T-cell maturation and a severe impairment of $CD8^+$ cells with an apparent bias toward γδ T-cell production (140).

These data have been published as Article on *Rivista Italiana di Genetica ed Immunologia Pediatrica, Journal of Neurological Science* and *Journal of Medical Genetics*, for the manuscripts see below.

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

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Abstract

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

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

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

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

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

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

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

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

1. Le immunodeficienze gravi combinate

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

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

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

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

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

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

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

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

2. La sindrome nude/scid

Dal fenotipo murino...

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

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

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

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

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

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

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

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

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

... al fenotipo umano

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

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

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

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

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

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

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

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

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

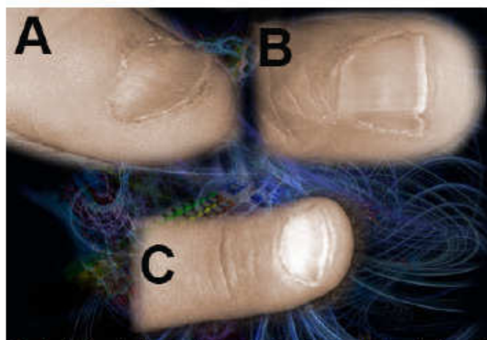
Dal punto di vista immunologico, i pazienti mostrano un difetto T selettivo testimoniato dall'assenza di risposta proliferativa associata ad un grave blocco nel differenziamento delle cellule T (2). In particolare, il fenotipo immunologico è caratterizzato da una drastica riduzione delle cellule CD3+,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

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

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

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

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



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

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

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

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

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

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

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ABSTRACT

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

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1. Introduction

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

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

2. Case report

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

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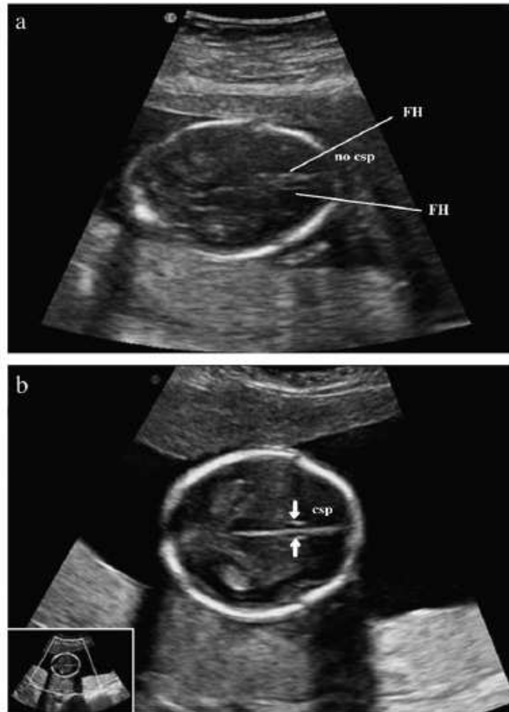

Fig. 1. (a) abdominal sonography does not reveal any structure on the cerebral midline to refer as CSP. Only medial wall of the frontal horn (FH) is evident. (b) sonography of a normal fetus at 16 weeks of gestation. The arrows indicate CSP.

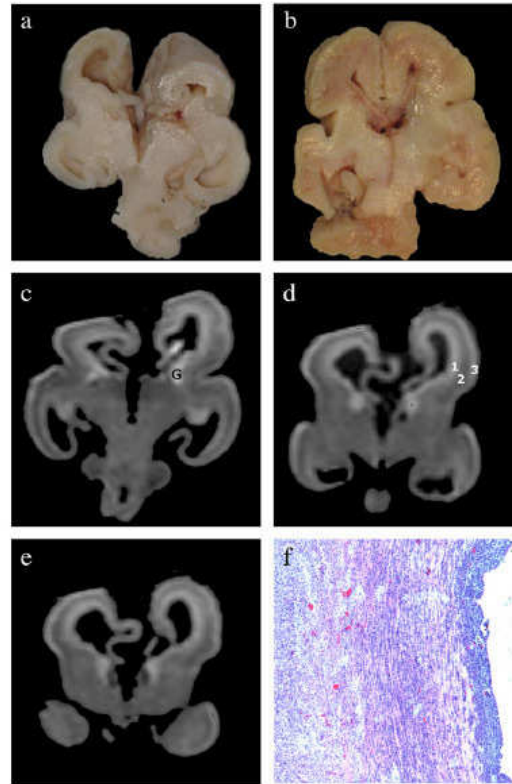

Fig. 2. (a) a coronal section of the brain, crossing the brainstem and the midbrain, in a Nude/SCID fetus revealed the abnormality in the development of corpus callosum. (b) a coronal section of the brain, in a control fetus of the same gestational age, revealed well formed midline structures with the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

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

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

3. Discussion

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

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

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

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SHORT REPORT

FOXN1 mutation abrogates prenatal T-cell development in humans

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ABSTRACT

Background The transcription factor FOXN1 is implicated in the differentiation of thymic and skin epithelial cells, and alterations in it are responsible for the Nude/SCID phenotype. During a genetic counselling programme offered to couples at risk in a community where a high frequency of mutated FOXN1 had been documented, the identification of a human *FOXN1*$^{-/-}$ fetus gave the unique opportunity to study T cell development in utero.

Results Total blockage of CD4$^+$ T cell maturation and severe impairment of CD8$^+$ cells were documented. Evaluation of the variable-domain β-chain (Vβ) families' usage among T lymphocytes revealed that the generation of T cell receptor (TCR) diversity occurred to some extent in the *FOXN1*$^{-/-}$ fetus, although it was impaired compared with the control. A few non-functional CD8$^+$ cells, mostly bearing TCRγδ in the absence of CD3, were found.

Discussion FOXN1 is crucial for in utero T cell development in humans. The identification of a limited number of CD8$^+$ cells suggests an extrathymic origin for these cells, implying FOXN1-independent lymphopoiesis.

The genetic study of human severe combined immunodeficiency (SCID) has clarified important issues concerning the rules that govern lymphocyte development and function.[1] The thymus has been long and unanimously considered the unique primary lymphoid organ where ontogeny of T cells, which are the essence of the cellular immune system, occurs.[2] The epithelial component of the thymic stroma is essential for T cell development.[3] In humans, DiGeorge syndrome has long been considered the prototype of an athymic disorder, even though in these patients some mature type T cells are present, suggesting the presence of a thymic rudiment or the presence of an extra-thymic site of lymphopoiesis. In mice and rats, spontaneous mutations in forkhead box-N1 (*Foxn1*) transcription factor gene, mapping on chromosome 11, revealed that Foxn1 is essential for thymic and skin epithelial differentiation and thymopoiesis.[4] This model is referred to as the Nude phenotype because of hairlessness.[5] These nude mutant animals develop an abnormal thymus, resulting in severe and selective T cell deficiency and an overall severely impaired immune system.[6] In particular, thymus-dependent lymphoid cells in the paracortex of lymph nodes and periarteriolar regions of the spleen are lacking.[7] These mice also fail to develop increased cellularity of lymph nodes draining a local injection of phytohaemagglutinin (PHA).[8] The human equivalent of the mouse Nude/SCID was first described in two sisters who originated from a geographically isolated community in the south of Italy, with the R255X homozygous mutation in the *FOXN1* gene.[9] This mutation leads to the complete absence of a functional protein similar to the previously described rat and mouse Foxn1 mutations.[10] In humans, *FOXN1* is located on chromosome 17. Identification of a number of heterozygotes for the same mutation in the village where the first patients originated suggested the need to offer to that population a prenatal diagnosis programme for the disease.

We report on an athymic Nude/SCID fetus, who gave the unique opportunity to gain further insights into the prenatal ontogeny of T lymphocytes in humans.

METHODS AND RESULTS

During the genetic counselling programme, a fetus at risk was identified. At 11 postmenstrual weeks of gestation, prenatal diagnosis was performed through villocentesis. Genomic DNA was extracted by standard procedures from chorionic villi and processed. *FOXN1* gene analysis was performed by a PCR assay and direct sequencing as previously described.[11] Analysis of the *FOXN1* gene sequence revealed the homozygous C-to-T transition at nucleotide position 792, leading to the nonsense R255X mutation. This finding led to interruption of the pregnancy. Analysis of protein expression by immunohistochemistry on fetal skin fragments revealed the absence of FOXN1 protein, as expected on the basis of the molecular alteration.

The concomitant assembly of CD4 and CD8 molecules on the thymocyte surface and their individual expression in conjunction with CD3 are markers of discrete stages of T cell development. In the immunophenotype study, cord blood mononuclear cells (CBMCs) were isolated from a Nude/SCID and a control human fetus, matched for gestational age, by density gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany). Cells were stained with the appropriate antibody (CD45-APC, CD3-PerCP, CD19-PerCP, CD16CD56-PE-Cy7, CD8α-PE-Cy7, CD4-FITC, TCRαβ-FITC, TCRγδ-PE, CD45RA-PE) (BD Pharmingen, San Diego, CA, USA) at 4°C for 30 min, washed and finally analysed using a FACSCanto II flowcytometer (Becton Dickinson, San Jose, CA, USA). Evaluation of Nude CBMCs revealed that the lack of thymus had led to a very low number of CD3$^+$

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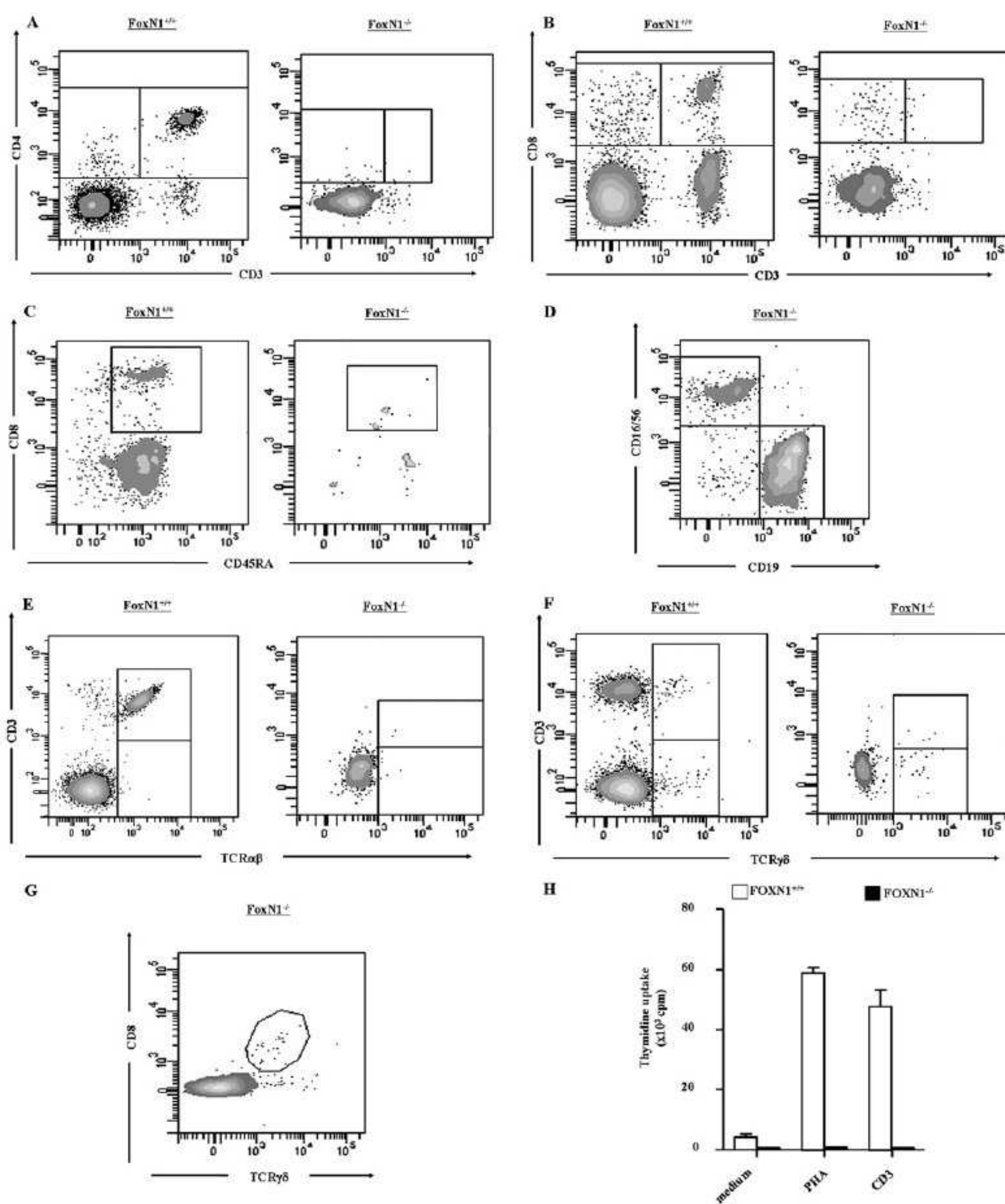

Figure 1 (A, B) Dot plots showing expression of CD3 and CD4 or CD3 and CD8 surface markers in the *FOXN1*$^{-/-}$ fetus and in the cord blood of a control of the same gestational age. (C) Expression of CD8 and CD45RA on CD3^{+} gated cells in the *FOXN1*$^{-/-}$ and control cord blood. (D) Dot plots showing expression of CD19 and CD16/56 surface markers in the *FOXN1*$^{-/-}$ fetus. (E, F) Dot plots showing expression of CD3 and T cell receptor (TCR)αβ or CD3 and TCRγδ on cord blood cells of the *FOXN1*$^{-/-}$ and control fetuses. (G) Dot plot showing expression of CD8 and TCRγδ in the CD3^{-} cells in the *FOXN1*$^{-/-}$ fetus. (H) Proliferation of cord blood mononuclear cells from *FOXN1*$^{-/-}$ and control fetuses after phytohaemagglutinin (PHA) stimulation or CD3 cross-linking. Incorporation of tritiated thymidine was evaluated after 4 days of culture.

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cells (3.5% vs 25.4% in the control). However, most of these cells in the *FOXN1*$^{-/-}$ fetus had a dim fluorescence intensity (figure 1A, B), suggesting a lower number of CD3 molecules per cell, indicating maturation arrest. In the *FOXN1*$^{-/-}$ fetus, 1.3% of the CD45$^+$ gated lymphocytes expressed CD4, whereas in the control fetus, this population was higher (23.4%). Of note, no CD4$^+$ cells co-expressed CD3 (0.3% vs 22% of the control fetus) (figure 1A). In contrast, in the *FOXN1*$^{-/-}$ fetus, we found a considerable number of CD8α^+ cells (10.3%), although CD8 cells co-expressing CD3 were very scarce (1.5% of the CD45$^+$ gated lymphocytes), whereas the proportion of CD3$^+$CD8$^+$ cells in the control was 5.9% (figure 1B). Of note, within the CD3$^+$CD8$^+$ subset, 1.1% and 4.5% of the CD45$^+$ gated lymphocytes displayed a naive phenotype, as assessed by the expression of the CD45RA isoform, in the *FOXN1*$^{-/-}$ fetus and in the control, respectively (figure 1C). Moreover, in the control, 2.4% of CD3$^+$ CBMCs were double positive for CD4$^+$ and CD8$^+$ markers. No double-positive T cells were found in the *FOXN1*$^{-/-}$ fetus.

As T cells originate from a common multipotent haematopoietic precursor cell, to exclude direct involvement of haematopoietic precursor cells in the alterations observed, CD34$^+$ cells were evaluated and found to be comparable to the control fetus, accounting for 1% of the CD45$^+$ haematopoietic cells. Consistent with this finding, no abnormalities in the development of B (65%) and natural killer (NK) (25%) cells were found in the *FOXN1*$^{-/-}$ fetus (figure 1D).

T cells are also distinguished by their cell surface T cell receptors (TCRs). A substantial reduction in T cells bearing TCR$\alpha\beta$ was observed in the *FOXN1*$^{-/-}$ fetus (1.5% vs 37.1% in the control) (figure 1E). In contrast, in the *FOXN1*$^{-/-}$ fetus 6.5% of the CD45$^+$ gated lymphocytes expressed TCR$\gamma\delta$, but the majority of these cells were CD3$^-$ cells (4.9%) (figure 1F). All these cells co-expressed the CD8$\alpha\beta$ heterodimer (figure 1G). In the control, TCR$\gamma\delta$ cells made up 3.7% of the CD45$^+$ gated lymphocytes and only a minority was CD3$^-$ (1.1%) (figure 1F).

The proliferation of CBMCs derived from Nude/SCID and control fetuses was determined from incorporation of tritiated thymidine during 72 h of culture after stimulation with 8 μg/ml PHA or anti-CD3 monoclonal antibody (1 ng/ml or 0.1 ng/ml), previously precoated on tissue culture plates for cross-linking. As expected, *FOXN1*$^{-/-}$ CBMCs showed an absent proliferative response after stimulation with anti-CD3 or PHA compared with the control cells (figure 1H).

As antigen receptor gene rearrangement is a hallmark of ongoing development in T lymphocytes, studies in the *FOXN1*$^{-/-}$ and control fetuses were conducted using high-throughput analysis of the TCR repertoire. T cells were separated into CD4$^+$

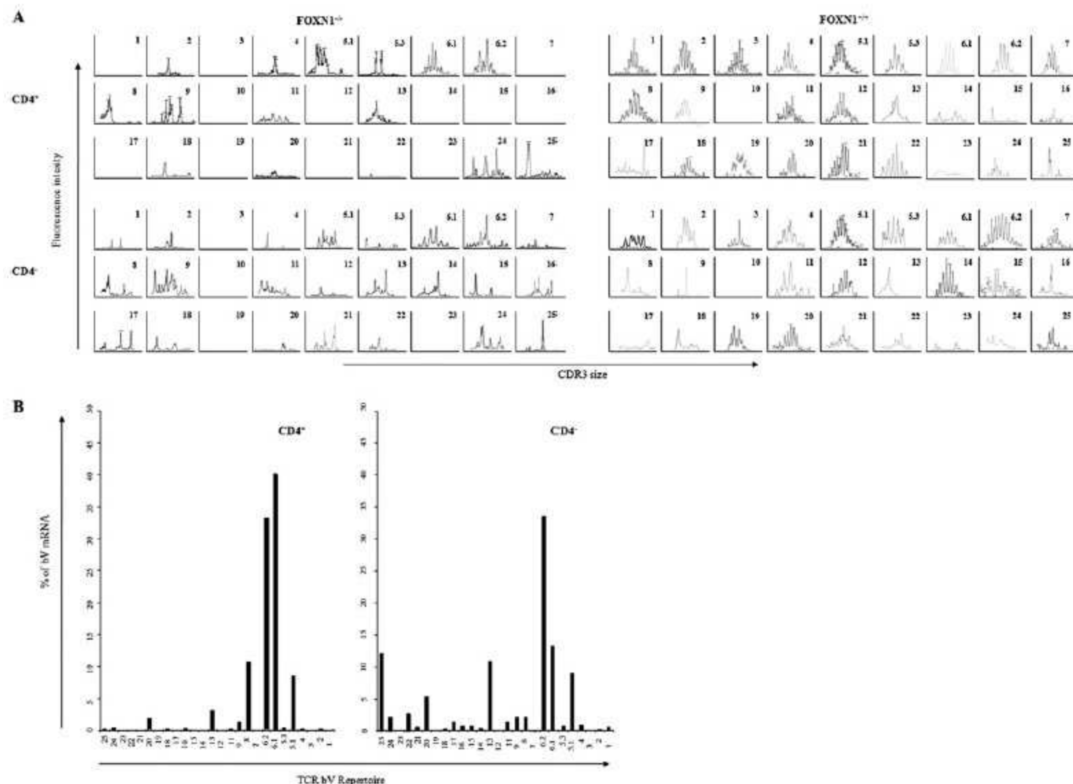

Figure 2 (A) Analysis of the T cell receptor (TCR) Vβ family repertoire in the cord blood mononuclear cells of *FOXN1*$^{-/-}$ and control fetuses. Results were obtained using primers that amplify CDR3 regions from the TCR Vβ genes indicated. Vβ families were considered normal if they showed five or more peaks in a Gaussian distribution. (B) The percentage of TCR Vβ expression within CD4$^+$ and CD4$^-$ cells in the *FOXN1*$^{-/-}$ fetus. The arrow on the x axis depicts TCR Vβ segments, and the arrow on the y axis shows the percentage of Vβ mRNA expression.

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and CD4$^-$ by magnetic sorting with coated beads (Dynabeads, Invitrogen, Carlsbad, CA, USA). For spectratype analysis, TCR CDR3β sequencing was performed after TCR variable-domain β-chain (Vβ) amplification with a common reverse primer (CB3 primer) and 27 different forward primers (TCR Vβ gene family primers). Results were analysed using CEQ 8000 software (Beckman Coulter). Evaluation of Vβ families usage revealed that the generation of TCR diversity was consistently impaired in the *FOXN1*$^{-/-}$ fetus, in contrast with the control of the same gestational age (figure 2A). Whereas in the control 26 of the 27 families were expressed in both subsets, in the *FOXN1*$^{-/-}$ fetus only a few families were expressed. Furthermore, two of them (Vβ 6.1 and 6.2) in the CD4$^+$ subset accounted for 73% of the whole repertoire (figure 2A, B), whereas in the CD4$^-$ subset only one family (Vβ 6.1) had a Gaussian profile (figure 2A). Moreover, in this subset, the Vβ 25 family exhibited an oligo-monoclonal profile (figure 2A). Contamination with maternal cells, which may have crossed the placenta and engrafted into the fetus in utero,[12] was ruled out by evaluating 15 highly polymorphic autosomal short-tandem repeat loci by multiplex-PCR (supplementary figure 1).

Thus, although the number of T lymphocytes is very low in the *FOXN1*$^{-/-}$ fetus, these data provide evidence that, in this model of congenital athymia, TCR gene rearrangement, although altered, occurs to some extent.

DISCUSSION

Our results provide evidence of the crucial role of FOXN1 in the early prenatal stages of T cell ontogeny in humans, in that its alteration leads to total blockage of CD4$^+$ T cell maturation and severe impairment of CD8$^+$ cells, with an apparent bias towards γδ T cell production. The different FOXN1-dependence of CD4$^+$ and CD8$^+$ cell maturation is similar to what occurs in the case of loss of the nuclear high-mobility group box protein, TOX, which in mice leads to selective blockage of CD4, but not CD8, differentiation.[13] Foxn1 is expressed in all thymic epithelial cells (TECs) during initial thymus organogenesis and broadly during fetal stages. Both fetal TEC differentiation and maintenance of the thymus microenvironment require 'cross-talk' between TECs and developing thymocytes.[14] Of note, in mice homozygous for the *Foxn1* gene mutations, TECs fail to differentiate, leading to blockage of thymopoiesis and severe immunodeficiency.[15]

Although extensive information on the role of the thymus in T cell development is available, some still unexplained evidence in human athymic conditions suggests that our in-depth knowledge of this process is lacking, in particular, about the involvement of different non-lymphoid tissues in T cell ontogeny. In human Nude/SCID, the absence of thymic tissue results in severe T cell immunodeficiency. As FOXN1 is selectively expressed in the thymus and skin, skin epithelial cells may have a role in productive T cell ontogeny, as previously shown in in vitro models.[16]

Our results show that FOXN1 is crucial for in utero T cell development, but not for B and NK cell differentiation in humans. The identification of a limited number of CD8$^+$ cells, bearing TCRγδ, suggests a different origin for these cells, although it is not known if they are derived from remnant thymus. However, this seems unlikely, as no thymic tissue at all was found in the autoptic evaluation. These cells are, however, unable to sustain a productive immune response into the periphery.

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Competing interests None.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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§2.3 Role of FOXN1 in Immune System

A critical role of the FOX transcription factors in the development of different tissues has been shown in a number of studies where FOX genes have been inactivated by gene targeting or mutations (141). These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions.

In particular, *FOXN1* gene, spanning about 30 kb (131, 142), is an epithelial cell-autonomous gene and is highly conserved in sequence and function in rodents and humans. The molecular mechanisms by which *FOXN1* expression and activity are regulated are only incompletely understood. Wnt proteins and bone morphogenetic proteins (BMPs) have been implicated in the transcriptional control of *FOXN1*. BMP4 has been shown to upregulate the expression of FOXN1, and FOXN1 might subsequently upregulate the expression of fibroblast growth factor (FGF) receptors (FGFRs), which in turn modulate the thymic stroma differentiation and thymopoiesis (143). Wnt proteins expressed by TECs and developing thymocytes can induce the expression of FOXN1. This seems to result from stabilization of β-catenin and it can be further regulated by PI3K through an undefined mechanism (**Figure 12**) (144). *In vitro* exposure of TECs to some Wnt is sufficient to upregulate FOXN1 protein expression in both an endocrine and paracrine fashion (145). Wnt belong to a large family of secreted glycoproteins that have important roles in cell-fate specification (143).

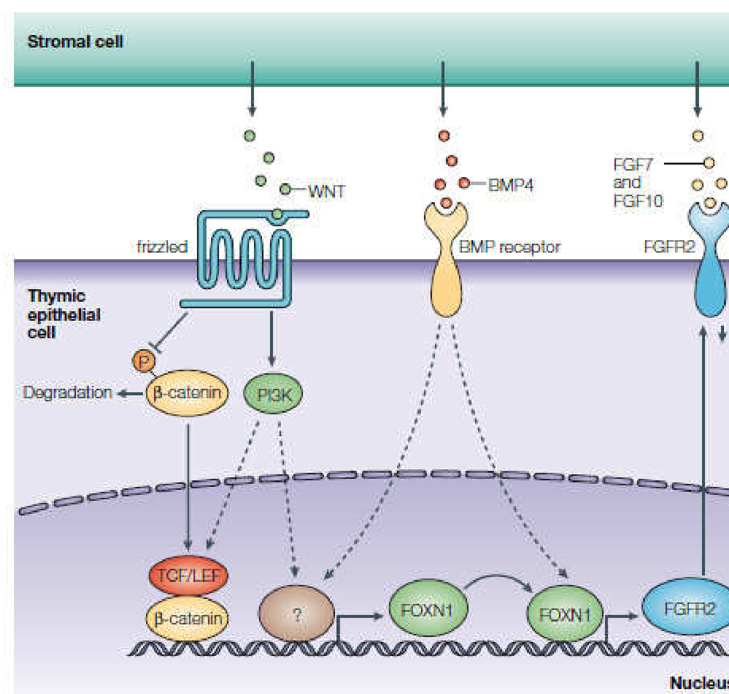

Figure 12. Thymic development and FOXN1

Foxn1 is expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation (117). Foxn1 exerts an important role (146) in inducing both cortical and medullary differentiation (147, 148). Although FOXN1 has been long 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.

§2.4 Conclusive remarks

Despite an extensive knowledge about the thymus role to foster T-cell development is available, some still unexplained evidence in human athymic conditions suggests that in-depth information of this process is still to be achieved and, in particular, the involvement of different non-lymphoid tissues in T-cell ontogeny. In human Nude/SCID, the absence of thymic tissue results in a severe T-cell immunodeficiency. Since FOXN1 is selectively expressed in the thymus and skin, skin epithelial cells could play a role for a productive T-cell ontogeny, as previously shown in *in vitro* models (149).

Our results demonstrate that FOXN1 is crucial for *in utero* T-cell development and not for B- and NK-cell differentiation in humans. The identification of a limited number of $CD8^+$ cells, bearing TCRγδ, suggests a different origin for these cells, which are however unable to sustain a productive immune response into the periphery. Thus, the expression of this factor in other tissue can be replacing the putative pivotal role of the thymus in maturation of thymocytes.

CHAPTER III

"Ataxia-Teleangiectasia due to alterations in ATM"

Ataxia-telangiectasia (A-T) is a rare recessive neurodegenerative disease that results from mutations in the ATM gene, resulting in diminished amounts or absence of ATM protein and/or ATM kinase activity (150). A-T is primarily a neurodegenerative disorder, whose underlying pathogenesis consists of a progressive cerebellar degeneration, mainly involving Purkinje and granule cells (151, 152). Neuronal degeneration is characterized by widespread loss of Purkinje cells in the cerebellum, atrophy of the cerebellar folia, granule cell loss and significant thinning of the molecular layer as revealed by autoptic and bioptic studies (153). Immunodeficiency is present in 60-80% of individuals with A-T, it is variable and do not correlate well with the frequency, severity or spectrum of infections (154, 155). The immunodeficiency is progressive and the most consistent immunodeficiency reported is poor antibody response to pneumococcal polysaccharide vaccines (154). Moreover, serum concentration of the immunoglobulins IgA, IgE and IgG2 may be reduced. Approximately 30% of individuals with A-T who have immunodeficiency have T-cell deficiencies.

Neither the normal function of ATM in the nervous system nor the biological basis of the degeneration in A-T has been extensively elucidated (150, 156). ATM is the central component of the signal-transduction pathway responding to DNA double-strand breaks (DSBs) caused by ionizing radiation, endogenous and exogenous DNA damage agents (157, 158). Following ATM activation, several DNA-repair and cell cycle checkpoint proteins are activated, leading to cell cycle arrest and DNA repair (159, 160). ATM plays a controlling role in recognition and repair of DNA damage, cell cycle arrest and cellular apoptosis by interacting with several downstream substrates (161). Thus, this disease is considered the prototype of DNA-repair defect syndromes (162). ATM is also involved in sensing and modulating intracellular redox status, even though it is not clear whether ATM is likely to prevent reactive oxygen species (ROS) production

(163). As for the molecular mechanisms of action of betamethasone, several lines of evidence indicate that steroids have remarkable effects through both nongenomic and genomic mechanisms, the latter well documented also in neural system (164, 165). The classical genomic mechanism of glucocorticoid action is cytoplasmic glucorticoid receptor (GR) mediated. Glucorticoids (GCs) bind and induce GR activation, followed by the GR translocation to nucleus and subsequent binding to glucocorticoid responsive element (GRE), thus modulating the transcription of a variety of genes including glucocorticoid-induced leucine zipper (GILZ) (**Figure 13**). GILZ is known as a marker GC transcriptional activity, rapidly induced by GC, able to regulate T lymphocytes activity, including T cell survival (166, 167).

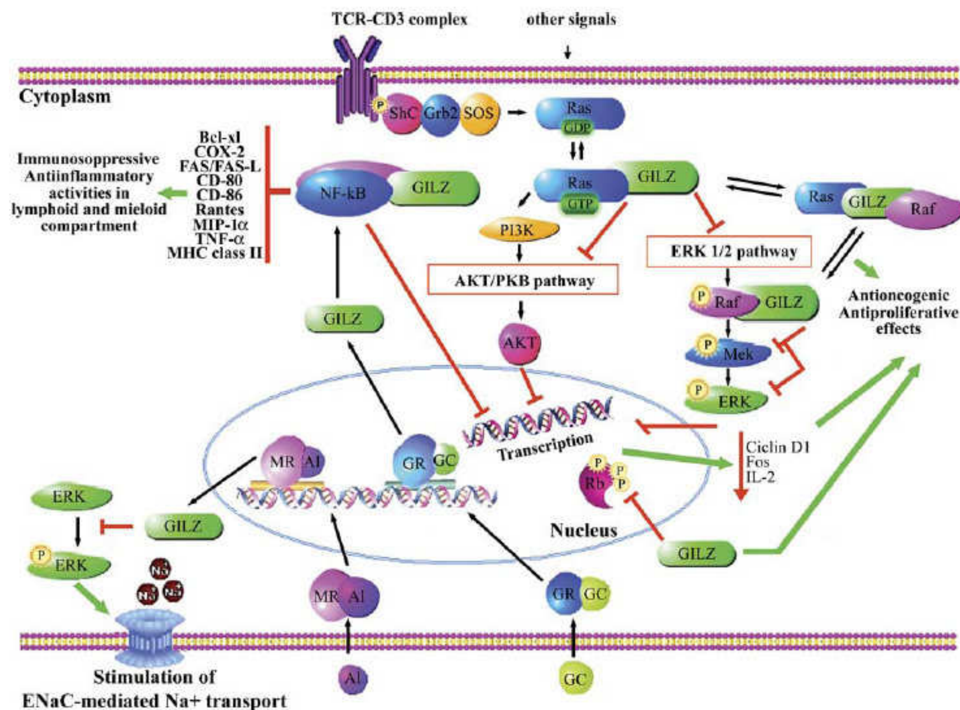

Figure 13. Glucorticoids response and GILZ signaling.

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

§3.1 Effects of steroid treatment in patients affected with Ataxia-Telangiectasia

Even though A-T is a non-curable disease that leads to a progressive neurodegenerative phenotype, a recent clinical report documented a drug-dependent improvement of neurological symptoms induced by a short course of oral betamethasone (168). This beneficial effect was inversely correlated with the severity of cerebellar atrophy, which is age-dependent (169).

We tried to determine the minimum-therapeutically-effective dosage of betamethasone, in order to reduce steroid-related side effects. The effect was still appreciable at a dosage as low as 0.01 mg/kg/day, corresponding to 10% of the full dosage. A long term study with low steroid dosage would also help define the benefit to risk ratio of such therapeutic intervention, to limit the concern on the immunosuppressive effect on the susceptibility to infections of these patients. Although this is an observational study on a limited number of affected patients, of note, in our study we found that GILZ expression in lymphocytes promptly increased in all patients during GC therapy at low dosage. Even though there is no evidence to prove that betamethasone may act in the brain, nevertheless, a recent study revealed the GILZ is expressed in specific areas of the brain and is upregulated in several brain regions of stress-exposed mice (170).

Our data indicate that betamethasone is effective in A-T at a minimal dosage and that GILZ may be a useful biomarker of the clinical response.

This study has been published as Article on *The European Journal of Neurology*, for the manuscript see below.

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Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia

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ataxia-telangiectasia, betamethasone, glucocorticoid-induced leucine zipper

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Background: Ataxia-telangiectasia (A-T) is a non-curable neurodegenerative disorder, associated with progressive neurological dysfunction, oculocutaneous telangiectasia, immunodeficiency, predisposition to cancer and radiosensitivity. A recent study documented improvement in neurological symptoms after a short-term therapy with betamethasone in patients with A-T.

Aim of this study was to evaluate the minimum therapeutically effective dosage of betamethasone on neurological symptoms of A-T.

Methods: Six responsive patients with A-T, received two 20-day cycles of oral betamethasone at 0.01 and 0.03 mg/kg/day (10% and 30% of the previously used full dosage), each followed by a 20-day washout period. Clinical and laboratory evaluations were carried out at T0 and at the end of each cycle. Neurological assessment was performed through the Scale for the Assessment and Rating of Ataxia (SARA). The glucocorticoid-induced leucine zipper (GILZ) and glucocorticoid receptor (GR) RNA expression were evaluated before and during the trial through real-time PCR.

Results: SARA scores significantly improved in all patients at the dosage of 0.03 mg/kg/day. In particular, three patients exhibited an improvement in 5/8 variables and two patients of 7 and 8 variables, respectively. Furthermore, the clinical improvement was already evident after the lower dosage. The basal GILZ and GR RNA expression were significantly lower in patients than in controls. GILZ expression increased in all patients after the beginning of the therapy, whereas no correlation between GR and the response was found.

Conclusion: Our data indicate that betamethasone is effective in A-T at a minimal dosage and that GILZ may be a useful biomarker of the clinical response. This study provides Class IIIA evidence that betamethasone at very low dosage is effective in improving neurological signs of patients affected with ataxia-telangiectasia.

Introduction

Ataxia-telangiectasia (A-T) is a rare recessive neurodegenerative disease that results from mutations in the ataxia-telangiectasia mutated (ATM) gene, resulting in diminished amounts or absence of ATM protein and/or ATM kinase activity [1]. The hallmark of A-T is the progressive neurological dysfunction characterized by uncoordinated and ataxic movements as a result of cerebellar atrophy or disfunction [2]. Other features of A-T include telangiectasia, immune and endocrine dysfunctions, cellular radiosensitivity, genomic instability, premature ageing and predisposition to cancer [2]. Neuronal degeneration is characterized by widespread loss of Purkinje cells in the cerebellum, atrophy of the cerebellar folia, granule cell loss and significant thinning of the molecular layer as revealed by autoptic and bioptic studies [3].

ATM is the central component of the signal transduction pathway responding to DNA double-strand breaks caused by ionizing radiation (IR), endogenous and exogenous DNA damaging agents [4,5]. Following ATM activation, several DNA repair and cell cycle checkpoint proteins are activated, leading to cell cycle arrest and DNA repair [6,7]. ATM plays a controlling

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role in recognition and repair of DNA damage, cell cycle arrest and cellular apoptosis by interacting with several downstream substrates [8]. Thus, this disease is considered the prototype of DNA-repair defect syndromes [9].

Ataxia-telangiectasia mutated is also involved in sensing and modulating intracellular redox status, even though it is not clear whether ATM is likely to prevent reactive oxygen species (ROS) production [10]. Previous studies have documented that the expression of glucocorticoid-induced leucine zipper (GILZ) is induced by glucocorticoids, following their interaction with cytoplasmic receptor (GR), thus being involved in the biological effect of steroids [11–13].

To date, A-T remains an incurable disease that leads relentlessly to death around the third decade of life [14,15]. A recent clinical report documented a drug-dependent improvement in neurological symptoms induced by a short course of oral betamethasone [16]. This beneficial effect was inversely correlated with the severity of cerebellar atrophy, which is age-dependent [17].

To reduce steroid-related side effects, aims of this study were both to evaluate the minimum therapeutically effective dosage of short-term oral betamethasone in patients with A-T and to define potential biomarkers of the clinical response.

Methods

Patients

Six patients with A-T (three males and three females) were selected because they were responsive to a full betamethasone dosage of 0.1 mg/kg/day and enrolled into this study. The patients received a diagnosis of A-T according to the European Society of Immunodeficiencies (ESID) criteria. A previous molecular study revealed a mutation of ATM in all patients, resulting in the absence of the protein in five patients and a decrease to 20% in the remaining patient (P2) (Table 1).

Study design

The patients, upon informed consent, received two subsequent 20-day cycles of oral betamethasone at the dosage of 0.01 and 0.03 mg/kg/day, divided every 12 h, corresponding to 10% and 30% of the dosage used in a previous study. The two cycles were separated by an off-therapy 20-day period as a washout phase. Clinical and laboratory evaluations were carried out at baseline (T0), at the end of the first cycle of therapy with 0.01 mg/kg/day (T20), before beginning the second cycle of therapy (T40), at the end of the second cycle of therapy with 0.03 mg/kg/day (T60) and 20 days after the withdrawal (T80). Neurological assessment was performed by means of the Scale for the Assessment and Rating of Ataxia (SARA) (see appendix E1 on the Neurology Web site at http://www.neurology.org/cgi/content/full/66/11/1717/DC1) [18]. Each clinical neurological examination was videotaped in the presence of two paediatric neurologists. A third physician reviewed the videotape blinding. The final results were obtained through the comparison of the individual evaluators' scores. During the treatment period, all patients underwent general physical examinations whilst potential adverse effects were being monitored through routine laboratory tests, including blood cell count, serum levels of transaminases, plasma electrolyte levels, renal functionality. Furthermore, weight and blood pressure were measured. The protocol was formally approved by the Ethical Committee for Biomedical Activities of "Federico II" University (trial registration n. 185/08).

Table 1 Clinical and laboratory data of patients affected with A-T

	P1	P2	P3	P4	P5	P6
Sex	M	F	M	M	F	F
Age (year)	18	19	10	9	9	8
Age at onset of unsteadiness of gait (year)	2	2	2	2	4	2
Loss of ambulatory skills (year)	9	9	Still ambulant	Still ambulant	5	Still ambulant
Cerebellar ataxia	+ + +	+ + +	+ +	+ +	+ + +	+ +
Resting tremor	+ + +	+ + +	±	±	+ + +	±
Increased AFP levels	Present	Present	Present	Present	Present	Present
ATM mutation	1463G > A/ del exons 32–36	8629insC/ 8977C > T	717delCCTC/ 717delCCTC	97delC/ 2113delT	748C > T/ 4776 + 1G > T	381delA/ 6679C > T

A-T, ataxia-telangiectasia; ATM, ataxia-telangiectasia mutated; AFP, alpha-fetoprotein; ±, borderline; + +, moderate; + + +, severe.

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Immunological and molecular studies

Immunological studies included the evaluation of the *in vitro* proliferative response to mitogens with standard procedures on peripheral blood mononuclear cells (PMBC). Phytohemagglutinin (PHA), phorbol myristate acetate (PMA) and pokeweed mitogen (PWM) were used. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 mCi [^{3}H]thymidine (Amersham International, Brussels, Belgium) 8 h before harvesting. Twenty healthy age-matched subjects were used as controls.

Real-time polymerase chain reaction (RT-PCR) for GR and GILZ expression levels: expression of the human GILZ and GR genes was analysed by RT-PCR. mRNAs were extracted using Trizol (Invitrogen, Paisley, UK) from PBMC. RT-PCR was carried out using QuantiTect Reverse Transcription (Qiagen, Hilden, Germany). For real-time PCR, sense primer for GILZ was 5′-AGGGGATGTGGTTTCCGTTA-3′ and antisense 5′- TGGCCTGTTCGATCTTGTTG-3′. For GR, sense: 5′- AACTGGAATAGGTGCCAAGG -3′; antisense: 5′- GAGCTGGATGGAGGAGAGC -3′. For HPRT, sense: 5′- TGGCGTCGTGATTAGTGATG-3′, antisense: 5′- GCACACAGAGGGCTACAATG -3′. PCR was carried out in CHROMO 4 (MJ Research Bio Rad, Milan, Italy) using DyNAmo HS SYBR GREEN qPCR kit (Finnzymes; Celbio, Siziano, Pavia, Italy). Relative amounts of GILZ, L-GILZ and GAPDH mRNA were calculated by the comparative ΔΔC(t) method. C(t) values were determined using the Opticon Monitor 2 software (MJ Research Bio Rad).

Statistical analysis

All data were expressed as mean ± standard deviation. A paired *T*-test was used for comparison of the total SARA scores and levels of GILZ expression between different times. Inter-rater agreement was calculated through weighted kappa coefficient. Moreover, to assess the reliability of SARA score, we also evaluated intraclass correlation coefficient (ICC). Values of $P < 0.05$ were considered statistically significant. The calculations were performed using the commercial statistical software MedCalc (MedCalc Software, Mariakerke, Belgium).

Results

Neurologic assessment and SARA scores

At the beginning of the study, three patients (P1, P2, P5) showed severe cerebellar ataxia and resting tremor, whilst the other three (P3, P4, P6) had milder neurological alterations. The clinical and laboratory features of all patients are summarized in Table 1. Neurological evaluation during betamethasone treatment revealed that SARA scores substantially improved in all patients at the dosage of 0.03 mg/kg/day (mean values ± SD of total SARA score at T60 and T40 were 17.5 ± 6.5 and 23 ± 5.2, respectively; $P < 0.05$). In particular, as indicated in Fig. 1, three patients exhibited an improvement in 5/8 variables whilst two patients improved in 7 and 8 variables, respectively. Only one patient showed improvement in 3 variables. However, clinical benefits were already evident at T20, corresponding to a betamethasone dosage of 0.01 mg/kg/day (mean values ± SD of total SARA score at T20 and T0 were 20.3 ± 5.5 and 23 ± 5.6, respectively; $P < 0.05$). In all cases, during the washout periods, a worsening of neurological functions was documented. At the end of the first washout period, in three patients, the SARA score was higher than at the beginning of the study. However, at the end of the study it returned below the T0 value in 2 of them. Two parameters, namely stance and gait, substantially improved at the lower dosage in patients with moderate or mild ataxia (P3, P4 and P6) whilst they did not change at all in patients with severe ataxia (P1, P2 and P5). We also noted that in one case (P5, 9 years of age) SARA scores were not adequate to document clinical improvement. In fact, the evaluation of the better gait quality (reduced motor awkwardness, motor instability, postural hypotonia, tremor and myoclonus) could not be properly recorded because of

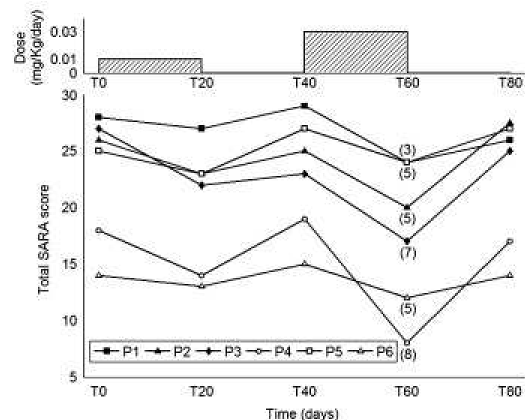

Figure 1 Changes of total SARA score during the steroid trial. In the upper side of the figure, the betamethasone dosage and washout periods are indicated. The behaviour of total SARA score during the study is illustrated in the six patients. Within the brackets, the number of SARA scale items that improved is expressed for each patient. SARA scale score significantly improved in all patients at the dosage of 0.03 mg/kg/day, but the amelioration was still appreciable at the dosage of 0.01 mg/kg/day.

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Table 2 Inter-rater reliability and agreement

Item	Inter-rater reliability (ICC) (n=3)	Inter-rater agreement weighted kappa coefficient U2/B	U1/B
1. Gait	0.995	0.894	0.927
2. Sitting	0.849	0.88	0.766
3. Hell-shine	0.793	0.664	0.687
4. Stance	0.991	0.94	0.94
5. Finger chase	0.909	0.834	0.751
6. Nose-finger	0.925	0.88	0.824
7. Fast-alternating movement	1.00	1.00	1.00
8. Speech disturbance	0.952	0.94	0.878
Total SARA score	0.988	0.901	0.879

ICC, Intraclass correlation coefficient.

the need of strong support for gait. Moreover, whilst on treatment the patient was able to stand with support, to eat and to drink alone, to write better and to colour within paint limits. All these performances could not be reached without steroid therapy. Moreover, the following three items: speech disturbance, finger chase and heel-to-shin slide were the ones that showed the most significant improvement in all patients. Overall, we observed that 33/48 variables (68%) examined in this study improved at T60 and 15/48 (31%) at T20 during steroid therapy. Inter-rater agreement was calculated through weighted kappa coefficient. The comparison of the scores between each unblinded (U1 and U2) and blinded (B) evaluator showed an "almost perfect agreement" (>0.81) in 8/9 (U1 vs. B) and 6/9 (U2 vs. B) items, respectively (Tables S1–S3). The other items showed 'substantial agreement' (0.61–0.80) (Table 2) [19]. Moreover, to assess the reliability of SARA score, we also evaluated intraclass correlation coefficient (ICC). Most single item had good inter-rater reliability with ICCs (>0.80) (Table 2). No alteration of alpha-fetoprotein (AFP) was noted during the study, and no side effects of steroids were documented. During the trial, no patient showed any known side effect of steroid therapy as diabetes mellitus, cataract, growth failure, osteoporosis and opportunistic infections.

GILZ and GR expression levels through RT-PCR studies

The evaluation of GR RNA expression documented that in all patients it was significantly lower than controls as shown in Fig. 2a ($P < 0.05$). A non-significant trend to an increase of GR expression between T40 and T60 was noted (data not shown). However, no correlation between GR expression and the clinical response to steroid therapy was observed. Similarly, basal GILZ RNA expression was significantly lower in patients than in controls as illustrated in Fig. 2a ($P < 0.05$). An

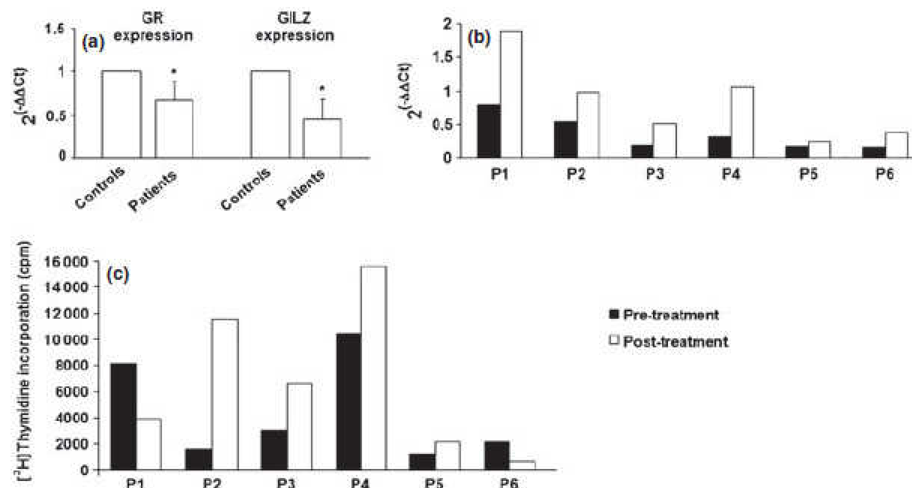

Figure 2 Relative GR and glucocorticoid-induced leucine zipper (GILZ) expression and proliferative response of peripheral blood mononuclear cells. (a) GR and GILZ RNA expression evaluated through real-time quantitative PCR in patients and controls before the treatment. Bars indicate the mean ± SD. * indicates $P < 0.05$ evaluated by T-Student test. (b) Comparison of GILZ RNA expression evaluated through real-time PCR in the six patients. Solid and white columns indicate pre- and post-treatment values, as illustrated. The betamethasone dosage was 0.01 mg/kg/day. (c) Proliferative response of peripheral blood mononuclear cells to PHA. Proliferation was evaluated pre- and post-treatment through [^{3}H]thymidine incorporation and expressed as cpm. In 4/6 patients, the proliferative response to the PHA increased during steroid therapy.

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increase in GILZ expression was observed after the first cycle of therapy in all patients ($P < 0.05$) (Fig. 2b) and was followed by a decrease to the baseline value during the washout period. Differently, during the second cycle, GILZ expression increased in three patients and was unchanged in the remaining patients.

Immunological evaluation

Lymphocytes count was evaluated at the different times (data not shown). In two of six patients (P1 and P4), the number of lymphocytes increased at T20 and T60 (from 1170 to 2330 and 1880 cells/mm^3; from 850 to 1390 and 1370 cells/mm^3). Moreover, the proliferative response to mitogens was evaluated at the same time. In four of the six patients, the proliferative response to the PHA increased by 33–85% during steroid therapy at T60 (Fig. 2c). The proliferative response to PMA or PWM paralleled the PHA response. However, the proliferative response remained below the mean control values ±2 SD of our laboratory.

Discussion

Even though A-T is a non-curable disease that leads to a progressive neurodegenerative phenotype, we recently demonstrated a drug-dependent improvement in cerebellar functions in a few patients with A-T after a short-term oral betamethasone course [16]. In this study, we tried to determine the minimum therapeutically effective dosage of betamethasone, to reduce steroid-related side effects. We noted that a drug dosage of 0.03 mg/kg/day, corresponding to 30% of the dosage proven useful in the previous study, induced a remarkable improvement in neurological function similar to that obtained using full dose betamethasone. Moreover, the effect was still appreciable at a dosage as low as 0.01 mg/kg/day, corresponding to 10% of the full dosage, thus indicating the possibility of a significant reduction of side effects related to steroid therapy. Since the steroid effect was very rapid, we cannot rule out that it represents a non-specific effect, not related to the pathogenesis. As previously shown, a worsening of the neurological signs was noted after the washout periods. It would be important to define in the near future whether a prolongation of the treatment will lead to the persistence of the neurological improvement. A long-term study with low steroid dosage would also help define the benefit to risk ratio of such therapeutic intervention, to limit the concern on the immunosuppressive effect on the susceptibility to infections of these patients.

Ataxia-telangiectasia is primarily a neurodegenerative disorder, whose underlying pathogenesis consists of a progressive cerebellar degeneration, mainly involving Purkinje and granule cells [20,21]. Neither the normal function of ATM in the nervous system nor the biological basis of the degeneration in A-T has been extensively elucidated [1,22]. Although ATM seems to be neuroprotective in the tissue undergoing oxidative stress and apoptosis, the intimate molecular mechanism of its property is still uncertain. A deregulation of intracellular oxidative stress has been consistently associated with various neurodegenerative conditions such as A-T [10]. Indeed, in Atm−/− mice *in vitro* survival of cerebellar Purkinje was significantly reduced, and most neurons exhibited dramatically reduced dendritic branching. These conditions were prevented by antioxidant drug administration, thus providing strong evidence that oxidative stress contributes for a phenotype defect in the cerebellum of Atm−/− mice [23,24].

As for the molecular mechanisms of action of betamethasone, several lines of evidence indicate that steroids have remarkable effects through both non-genomic and genomic mechanisms, the latter well documented also in neural system [25,26]. The classical genomic mechanism of glucocorticoid action is cytoplasmic glucocorticoid receptor (GR) mediated. GC binds and induces GR activation, followed by the GR translocation to nucleus and subsequent binding to glucocorticoid responsive element (GRE), thus modulating the transcription of a variety of genes including GILZ. GILZ is known as a marker GC transcriptional activity, rapidly induced by GC, able to regulate T lymphocytes activity, including T-cell survival [27,28]. Although this is an observational study on a limited number of affected patients, of note, in our study we found that GILZ expression in lymphocytes promptly increased in all patients during GC therapy at low dosage. Even though there is no evidence to prove that betamethasone may act in the brain; nevertheless, a recent study revealed the GILZ is expressed in specific areas of the brain and is up-regulated in several brain regions of stress-exposed mice [29]. In addition, brain areas related to motor and sensory systems and to cerebellar functions widely express GILZ. Previous studies indicated that also GR is abundantly and broadly expressed in the brain including the prefrontal cortex, hippocampus, Purkinje cell layer of the cerebellum [30]. Thus, GILZ could be implicated in a variety of roles in the brain including neural activation and transmitter release, motor system and stress response–related roles [30].

In the immune system, the immunosuppressive and anti-inflammatory effects of GC are because of the modulation of either innate or adaptive immunity, eventually resulting in the inhibition of T-lymphocyte activation and proliferation, cytokines production and

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transactivation of several transcription factors [31–33]. Of note, in our study, we observed a paradoxical response of lymphocytes to steroid therapy in that both dosages of betamethasone, corresponding to 10% or 30% of the full dosage, increased the proliferative response to mitogens. Whether this paradoxical response to betamethasone in lymphocytes might be informative in elucidating the mechanism of action of steroids on Purkinje cells remains to be clarified. This effect on lymphocytes might be the hallmark of a direct steroidal effect in restoring the altered biological process responsible for cell damage.

Although additional randomized trials with a larger cohort of patients are required, our observational study suggests improvement in function over a short term at a low dosage. This would open a new window of intervention in this so far non-curable disease. It should be emphasized that the benefit to risk of a long-term treatment should be carefully evaluated because of the side effects of steroids, as diabetes mellitus, cataract, growth failure and osteoporosis. In addition, an increased risk of infections could be related to the steroid therapy. However, patients with A-T only have a limited increase in infection susceptibility [34]. In any case, new therapeutic strategies should be explored to minimize the undesired effects.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparison of GILZ RNA expression evaluated through real-time PCR in the 6 patients. Solid and white columns indicate T40 and T60 values, respectively.

Table S1 Unblinded 1.

Table S2 Unblinded 2.

Table S3 Blinded.

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§3.2 Conclusive remarks

Although additional randomized trials with a larger cohort of patients are required, our observational study suggests improvement in function over a short-term at a low dosage. This would open a new window of intervention in this so far non-curable disease. It should be emphasized that the benefit to risk of a long-term treatment should be carefully evaluated due to the side effects of steroids, as diabetes mellitus, cataract, growth failure and osteoporosis. In addition, an increased risk of infections could be related to the steroid therapy.

It would be important to define in the near future whether a prolongation of the treatment will lead to the persistence of the neurological improvement. A long-term study with low steroid dosage would also help define the benefit to risk ratio of such therapeutic intervention, to limit the concern on the immunosuppressive effect on the susceptibility to infections of these patients.

CHAPTER IV
"Immunodeficiencies associated with unidentified molecular defects"

Despite the enormous progress that has occurred in identifying molecular causes of immunodeficiency, many challenges remain.

Among the diseases for which the fundamental causes remain unknown there are the Hyper IgE syndrome (HIES) and recently, our group documented a phenocopy of the congenital form of SCID due to an inhibitory anti-lymphocytic autoantibody.

§4.1 Alteration of IL-12R signaling related with high serum levels of IgE

HIES is a very rare primary immunodeficiency, characterized by the high serum levels of IgE (>2000 IU/ml), recurring staphylococcal skin abscesses and pneumonia with pneumatocele formation. Most cases are sporadic, but both autosomal dominant forms of HIES and autosomal recessive forms have been described. Skeletal symptoms such as hyperextensibility of joints, scoliosis, osteoporosis, and retained primary teeth are associated with the autosomal dominant form. An autosomal recessive disease characterized by severe recurrent viral infections, extreme eosinophilia and devastating neurological complications that are often fatal in childhood, has been described. Patients with the autosomal recessive form appear to be prone to developing autoimmune diseases. HIES usually presents very early in life. Clinical diagnosis has been based on a profile of immunologic and non-immunologic features leading to a composite score. Specific mutations have not been identified in these patients (171).

It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance. A Th1 response is implicated under ordinary circumstances in resistance to several intracellular pathogens, but an excessive Th1 response is associated with different autoimmune diseases, as rheumatoid arthritis (172), type I diabetes (173) or multiple sclerosis (174). On the contrary, a Th2 dominated

response, usually involved in the response to extracellular pathogens as parasitic or helminths, is associated with allergic disorders and the progression of chronic infections as AIDS (175).

The dimeric cytokine IL-12, produced by B cells and macrophages, plays a pivotal role for the induction of a Th1 response (176). The receptor is upregulated during T-cell activation and IL-12Rβ2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rβ1 is constitutively expressed in resting cells (177). The transducing element of the receptor is the IL-12Rβ2 chain that functionally interacts with STAT4 (178). Th1 cells develop in the presence of interleukin IL-12 and STAT4 signaling and secrete mainly IFN-γ (179).

We investigated, at a functional level, whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus. In particular, the activation of STAT4 molecule, which follows IL-12R triggering, the analysis of gene transcription and membrane assembly of the receptor itself were investigated in allergic children divided on the basis of the amount of serum IgE. We provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction.

It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by HIES. However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features (171). In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed.

The results of this study were published as Article on *Cellular Immunology*. See below for the paper.

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

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ABSTRACT

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

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1. Introduction

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

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

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

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2. Materials and methods

2.1. Subjects

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

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

The study has been approved by the Institutional Review Board.

2.2. Cell culture and proliferation assay

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

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

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

2.3. Generation of Th1 cell lines

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

2.4. Analysis of STAT4 activation

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

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

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

2.6. Analysis of IL-12Rβ2 chain RNA expression

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

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

2.7. Statistical analysis

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

3. Results

3.1. Proliferative responses

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

3.2. Analysis of STAT4 tyrosine phosphorylation/activation

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

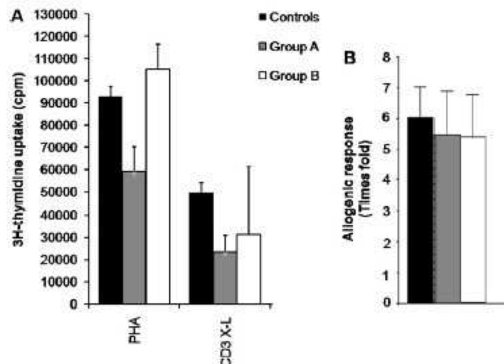

Fig. 1. Proliferative responses in patients and controls. Patients were divided on the basis of IgE levels (group A, $n = 10$, IgE >2000 kU/l; group B, $n = 10$, IgE value between the age specific mean ± 2 SD and 2000 kU/l; controls, $n = 20$). (A) Proliferative response to PHA (8 μg/ml) and CD3 cross-linking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

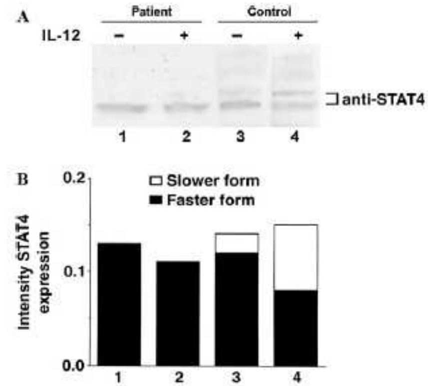

Fig. 2. Analysis by immunoblot of STAT4 protein in controls and patients with very high IgE levels (>2000 kU/l). (A) Representative experiment, out of 6, showing that rIL-12 stimulation induces in controls the appearance of a slow migrating phosphorylated form of the protein, whereas in patients only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

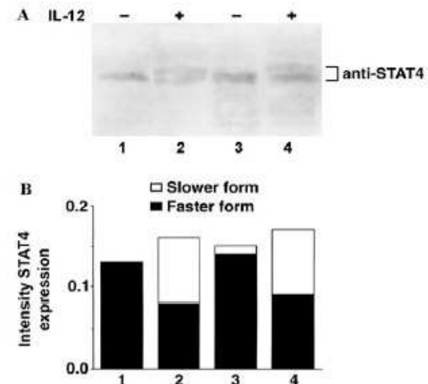

Fig. 3. Analysis by immunoblot of STAT4 protein in controls and patients with IgE values between the age specific mean ± 2 SD and 2000 kU/l. (A) Representative experiment, out of 3, showing that rIL-12 stimulation induces both in control and patient the appearance of a slower form of STAT4. PBMC were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

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

3.3. IL-12R expression on T cells

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

3.4. IL-12Rβ2 mRNA expression

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

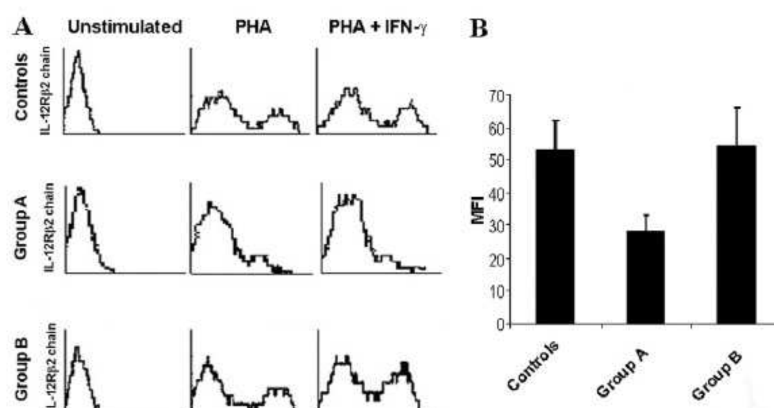

Fig. 4. Membrane expression of β2 chain of IL-12R on T cells. IL-12Rβ2 membrane expression on resting or T-cell blasts, induced by stimulation with PHA for 72 h in the absence or presence of IFN-γ, in controls and patients divided in two groups on the basis of IgE levels as indicated in Section 2. Dual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

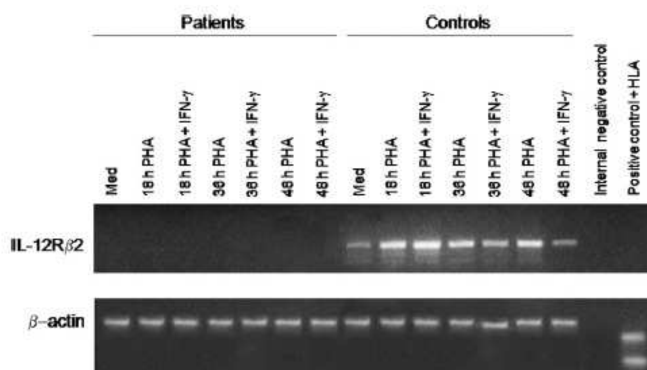

Fig. 5. mRNA expression of IL-12Rβ2 chain in controls and group A patients (IgE levels: >2000 kU/l). Representative experiment showing that in controls, β2 chain mRNA expression increased after 18 h of PHA stimulation. Lanes 1 and 8: freshly isolated PBMC. T-cell blasts were generated by 18, 36 and 48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

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

4. Discussion

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

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

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

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

Acknowledgment

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§4.2 Conclusive remarks

In this study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction. In particular, in this case we characterized an unappreciated relationship between an impairment of Th1 induction due to alteration of IL-12R signaling and high levels of serum IgE but in the absence of HIES.

However, it should be noted that abnormalities of IL-12 signaling pathway is not sufficient *per se* to cause an allergic disease in children. Our data could imply a pathogenic link between infections and allergy in children. In the light of this consideration, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help ameliorate the overall management of these patients. In fact, there is evidence that patients with severe forms of allergic manifestations are more susceptible to respiratory infections, and vice versa infections may trigger acute episodes of asthma (180).

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

§ 4.3 SCID-like phenotype associated with an autoantibody

Interestingly, evidence is emerging that, unlike total immunodeficiencies, partial T-cell immunodeficiencies are more frequently associated with hyperimmune dysregulation with a frank autoimmune phenotype (181). Even more interesting the observation that the loss of function or gain of function alterations in the immune system functionality may arise from abnormalities of the same multiple genes, that in some cases lead to total and in other to partial deficiencies. This would imply that the hyperimmune dysregulation is not related to selected genes alterations, but rather to the partial T-cell immunodeficiency itself (181).

We reported a patient with a clinical phenotype resulting in a typical lymphocytopenic $T^-B^+NK^+$ SCID. A similar phenotype is generally due to an impairment of 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 functionality (182-184).

The patient was born at 42 weeks of gestation to unrelated healthy parents. At 6 months of age the child was hospitalized because of chronic diarrhoea, dystrophic features and febrile seizures. At 8 months of age, the immunological evaluation revealed decreased IgG serum levels (<47mg/dl) and normal IgA and IgM. Moreover, the patient showed an autoimmune haemolytic anemia. At the time of the study, lymphocytes were 3 x 10^9/l, with 34.5% of $CD3^+$ cells, 25.5% and 15.3% of $CD4^+$ and $CD8^+$, 4% of $CD19^+$ and 15% of $CD56^+CD3^-$ cells. A severe lymphocyte functional impairment, in the absence of HIV or any other viral infection, was noted. Patient's PBMC exhibited absent proliferation to PHA. To identify a potential inhibitory factor, the patient's serum was added to PBMC from 5 distinct healthy controls. A significant higher inhibitory effect when compared to normal human serum was noted (**Figure 14**).

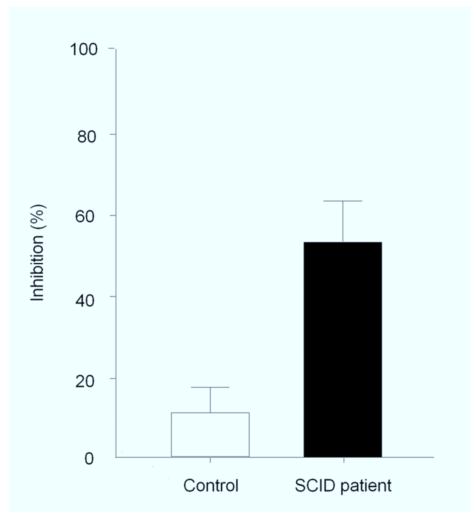

Figure 14. Inhibition (%) of normal peripheral mononuclear cells (PBMC) proliferation after stimulation with PHA (8 μg/ml). Cultures were performed in the presence of either 5% normal human serum or 5% SCID patient's serum. Bars show mean ±SD, n=5

To define the potency of the inhibitory effect, scalar doses of either SCID patient's serum or normal serum were used and a dose-response curve was obtained. A linear increase of the inhibition was observed, reaching the maximum inhibition at the 10% concentration (**Figure 15**).

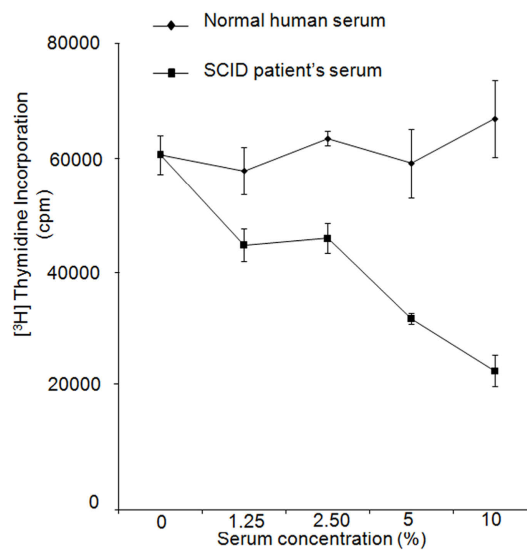

Figure 15. [^{3}H]thymidine incorporation by normal PBMC stimulated with PHA (8 μg/ml) and incubated with scalar concentrations (0, 1.25, 2.5, 5 and 10%) of either normal human serum or SCID patient's serum. Each point represents the mean ±SD, n=3.

Serum heat inactivation did not abolish the inhibitory effect on the proliferation to PHA of control PBMC (**Figure 16**), thus ruling out a role of the complement in the phenomenon.

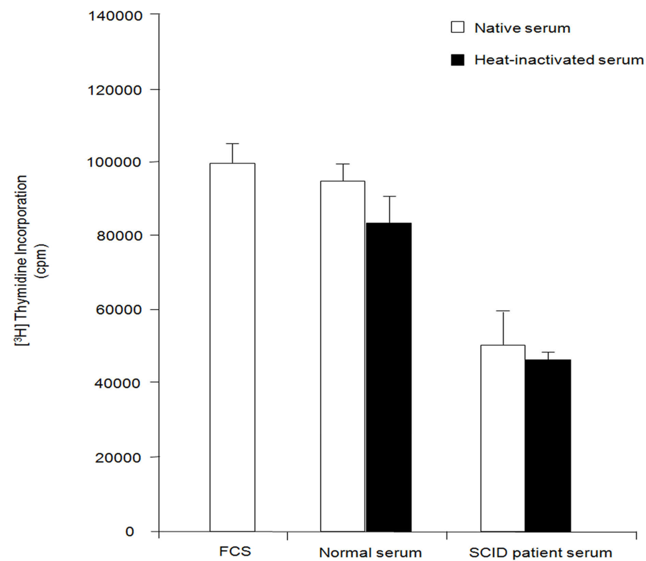

Figure 16. Effect of native and heat-inactivated serum on proliferative response by normal PBMC stimulated with PHA (8 µg/ml). Before use, both patient's and normal serum aliquots were heated at 56° C for 30 min to inactivate complement. PBMC were cultured in medium containing 10% native serum or heat-inactivated serum. Values are expressed as mean ±SD, n=3.

To evaluate whether the patient's serum inhibitory effect was attributable to an anti-lymphocyte autoantibody, affinity purified IgG, from both patient and control were tested for the inhibitory property. The patient's purified IgG fraction was able to significantly inhibit the proliferative response of normal PHA-stimulated PBMC differently from control purified Ig fraction (**Figure 17**).

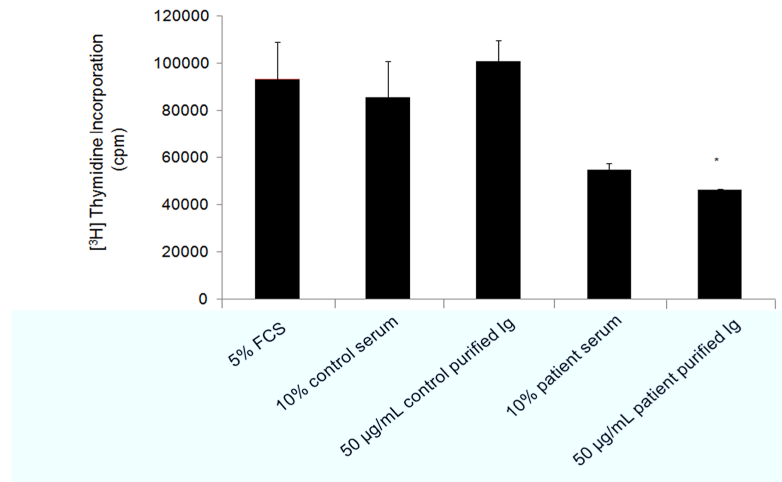

Figure 17. Inhibition by SCID patient's Ig of proliferative response by normal PBMC stimulated with PHA (8 µg/ml). Control PBMC were cultured in the presence of either 10% patient's native serum or patient's purified Ig (50 µg/ml) or in the presence of either 10% normal native serum or normal purified Ig (50 µg/ml), n=3. Negative control: FCS. Bars show means ±SD. * Statistically significant difference (P <.05) compared to the cultures containing control purified Ig fraction.

The patient also developed a severely progressive active autoimmune hepatitis, treated with azathioprine (AZA) (1.5 mg/kg/day) and steroids. A paradoxical effect of immunosuppression on cell subsets was noted in that, an increase of the $CD3^+$ and $CD4^+$ occurred. Differently, $CD19^+$ and $CD8^+$ cells didn't change significantly during the therapy (**Figure 18**).

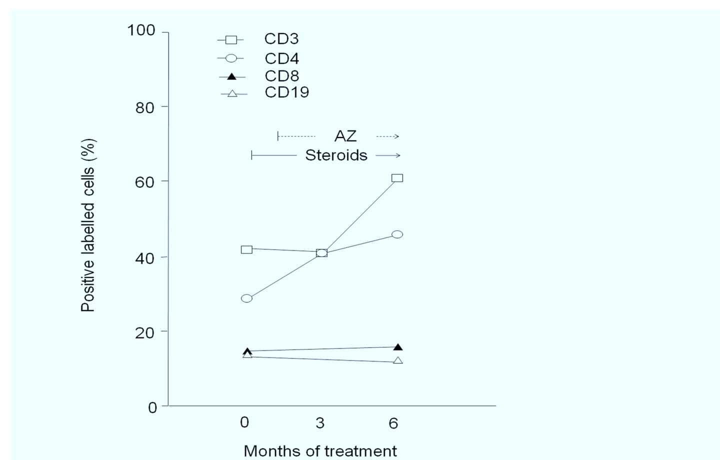

Figure 18. Increase in the percentage of major lymphocyte subsets in the SCID patient during the treatment with azathioprine (AZA) and steroids. PBMC from the SCID patient were stained using anti-CD3, anti-CD4, anti-CD8 and anti-CD19 antibodies and analyzed by fluorescence activated cell sorter (FACS). The horizontal lines indicate the period of immunosuppressive treatment with AZA (dotted line) and steroids (solid line).

In conclusion, this SCID-like patient was characterized by a severe T-cell activation deficiency, in whose serum an inhibitory factor, precipitated in the purified Ig fraction and able to potently inhibit control cells proliferation, was identified. So far, an autoreactive anti-lymphocyte antibody able to induce a SCID phenocopy has never been described.

The results of this study were published on *Journal of Investigational Allergology & Clinical Immunology*.

§ 4.4 Conclusive remarks

We documented in a SCID-like phenotype a novel pathogenetic mechanism due to an inhibitory anti-lymphocytic autoantibody, resulting in a total T-cell activation deficiency associated with autoimmunity. This complex phenotype represents a phenocopy of the congenital forms of SCID.

Our data indicate a direct role of the antibody as negative regulator of T-cell functionality. However, it is also possible that the inhibitory autoantibody in our patient is the consequence of a hyper immune dysregulation rather than the cause of the T-cell defect, whose genetic alteration still remains to be identified. Although the functional defect observed in our patient may theoretically be related to viral induced anergy (185), no viral infection was documented and the functional defect was total differently from what observed in viral induced anergy.

TECHNOLOGIES

§ Cells and cell cultures

Peripheral Blood Mononuclear cells (PBMC) were obtained from patients and healthy donors by Ficoll-Hypaque (Biochrom) density gradient centrifugation.

B lymphoblastoid cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMC using standard procedures.

The human 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.

Primary leukemic cell lines, consisting of acute lymphoblastic leukemia (ALL) cells, were obtained from aspirated bone marrow of 3 patients. Normal bone marrow cells were obtained from healthy donors and used as control cells.

Cord blood mononuclear cells (CBMCs) were isolated from Nude/SCID fetus and control human fetus, matched for the same gestational age, by density gradient centrifugation over Ficoll-Hypaque (Biochrom), after written consent. Cell suspensions were prepared in RPMI 1640 medium.

§ Genetic counseling

A genetic counseling program was offered to the village population from which the proband affected with the Nude/SCID phenotype was originated. Since *FOXN1* mutation is particularly devastating, because it leads to the absence of the

thymus and SCID, prenatal diagnosis by direct genetic analysis was performed for couples at risk.

§ A-T patients and study design.

Six A-T patients (3 males and 3 females) were selected because they were responsive to a full betamethasone dosage of 0.1 mg/kg/day and enrolled into this study. The patients received a diagnosis of A-T according to the European Society of Immunodeficiencies (ESID) criteria. A previous molecular study revealed a mutation of ATM in all patients, resulting in the absence of the protein in five patients and a decrease to 20% in the remaining patient (P2).

The patients, upon informed consent, received two subsequent 20-day-cycles of oral betamethasone at the dosage of 0.01 and 0.03 mg/kg/day, divided every 12 h, corresponding to 10 and 30 % of the dosage used in a previous study. The two cycles were separated by an off-therapy 20-day-period as a wash-out phase. Clinical and laboratory evaluations were carried out at baseline (T0), at the end of the first cycle of therapy with 0.01 mg/kg/day (T20), before beginning the second cycle of therapy (T40), at the end of the second cycle of therapy with 0.03 mg/kg/day (T60) and 20 days after the withdrawal (T80). Neurological assessment was performed by means of the Scale for the Assessment and Rating of Ataxia (SARA) (see appendix E1 on the Neurology Web site at http://www.neurology.org/cgi/content/full/66/11/1717/DC1). Each clinical neurological examination was videotaped in the presence of 2 pediatric neurologists. A third physician reviewed the videotape blinding. The final results were obtained through the comparison of the individual evaluators' scores. During the treatment period, all patients underwent general physical examinations while potential adverse effects were being monitored through routine laboratory tests, including blood cell count, serum levels of transaminases, plasma electrolyte levels, renal functionality. Furthermore, weight and blood pressure were measured. The protocol was formally approved by the Ethical Committee for Biomedical Activities of "Federico II" University (trial registration n. 185/08).

§ Subjects with elevated IgE levels

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

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

The study has been approved by the Institutional Review Board.

§ SCID-like patient's profile

The patient was born at 42 weeks of gestation to unrelated healthy parents. At 8 months of age, the patient showed an autoimmune haemolytic anemia and a progressive decline of $CD4^+$ cells, resulting in a typical lymphocytopenic form of severe combined immunodeficiency. At the time of the study, lymphocytes were 3 x 10^9/l, but a severe lymphocyte functional impairment in the absence of HIV or any other viral infection was first noted as above described. IL-2Rγ alterations were ruled out. Thereafter, 2 bronchopneumonia and an interstitial pneumopathy

occurred despite intravenous Ig replacement therapy and anti-infectious agents. Autoreactive antibodies toward smooth muscle, red and white cells were detected. During the follow-up, the patient developed a severely progressive active autoimmune hepatitis, diagnosed according the AIH international score, treated with Azathioprine (AZA) (1.5 mg/kg/day) and steroids. At 4 years of age the patient died of disseminated interstitial pneumopathy, while the search for a HLA-matched donor was still pending.

§ 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).

To evaluate allogeneic response in patients with elevated IgE levels, cells (1 x 10^5) were stimulated with an equal amount of irradiated stimulator cells from controls in a standard one-way mixed lymphocyte reaction assay.

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

Recombinant human GH (rGH) was obtained from Serono (Saizer 4). 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 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 HRPconjugated 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.

§ Brain alterations evaluation in Nude/SCID fetus

The abdominal sonography was performed through GE Voluson E8 Ultrasound Machine. Brain MRI study was performed at 3 Tesla (Magnetom TRIO, Siemens, Germany) using a 3D low-angle, gradient-echo sequence (TR/TE 572/3.7 msec, FA 9°, FOV150mm, acquisition matrix 144x256, slice thickness 600mm, using a quadrature volumetric coil) providing a T1w volume which was resliced along axial, coronal and sagittal planes. Images were processed using GIMP (http://www.gimp.org) to remove paraformaldehyde background.

§ PCR and quantitative real-time PCR analysis

After a written informed consent was obtained from parents of Nude/SCID fetus, genomic DNA was extracted by standard procedures from villous and processed. *FOXN1* DNA analysis was performed according to a polymerase chain reaction (PCR) assay previously described. Briefly, a PCR fragment containing exon 4 of the FOXN1 gene (formerly named exon 5) was amplified using the primers exon 5F: 5-CTTCTGGAGCGCAGGTTGTC-3 and exon 5R: 5-TAAATGAAGCTCCCTCTGGC-3. The PCR product was sequenced using an ABI prism 310 Genetic Analyzer (Applied Byosystems Inc.).

Total RNAs were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions.

To evaluate the effect of γc on cell survival and proliferation, 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). 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.

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'

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

To evaluate GR and GILZ expression levels, reverse transcriptase was done using QuantiTect Reverse Trascription (Qiagen). For real-time PCR, sense primer for GILZ was 5'-AGGGGATGTGGTTTCCGTTA-3', and antisense 5'-TGGCCTGTTCGATCTTGTTG-3'. For GR, sense: 5'-AACTGGAATAGGTGCCAAGG -3'; antisense: 5'-

GAGCTGGATGGAGGAGAGC -3'. For HPRT, sense: 5'-TGGCGTCGTGATTAGTGATG-3', antisense: 5'-GCACACAGAGGGCTACAATG -3'. PCR was done in CHROMO 4 (MJ Research Bio Rad) using DyNAmo HS SYBR Green qPCR kit (Finnzymes; Celbio). Relative amounts of GILZ, L-GILZ and GAPDH mRNA were calculated by the Comparative ΔΔC(t) method. C(t) values were determined using the Opticon Monitor 2 software (MJ Research Bio Rad).

To evaluate *IL-12Rβ2* chain expression, RNA was reverse transcribed into cDNA using Expand^{TM} 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.

§ Microsatellite analysis

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

§ Flow cytometry analysis

CBMC of Nude/SCID fetus were stained with the appropriate antibodies (CD45, CD3, CD8α, CD4, TCRαβ, TCRγδ, CD45RA (BD Pharmingen) on ice, washed and analyzed using a FACSCanto II flowcytometer Becton Dickinson. Data were analyzed using FACSDiva software.

The following conjugated monoclonal antibodies with appropriate isotype- and fluorochrome-matched isotype controls were used in one-colour or two-colour

staining: anti-CD3 (Leu-4), anti-CD8 (Leu-2a), anti-CD4 (Leu-3a) and anti-CD19 (Leu-12) (Becton Dickinson). After immunostaining, PBMC isolated from SCID-like patient were analyzed on a fluorescence activated cell sorter (FACSCalibur) (Becton Dickinson) using CellQuest (Becton Dickinson) software. PBMC were gated on the basis of linear forward and side scatter characteristics. Each lymphocyte subset was expressed as a percentage of positive cells.

§ Evaluation of TCR Vβ repertoire

T cells were separated into $CD4^+$ and $CD4^-$ cells by magnetic sorting with coated beads (Dynabeads). RNA was prepared with Trizol (Gibco) according to the manufacturer's instructions. For Spectratyping analysis, 400 ng of total RNA were retrotranscribed and amplified in a single-step reaction with the SuperScript™ III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). The same primers were used both for reverse transcription and for amplification. TCR CDR3β sequencing was performed after TCR β-chain amplification with a common reverse primer (CB3 primer) and 27 different forward primers (TCR Vβ gene family primers), as described. An initial step of reverse transcription at 45°C for 30 min was followed by amplification, for a total of 35 cycles. Cycling conditions were: 30 s at 94˚C for denaturation, 30 s at 55˚C for annealing, and 30 s at 72˚C for the extension with a final extension of 10 min at 72˚C. PCR products were then run on a CEQ 8000 automatic capillary sequencer (Beckman Coulter) and fractionated on the size of the CDR3 region, as described. Results were analyzed using CEQ 8000 software (Beckman Coulter). Vβ families were considered normal when showing 5 or more peaks with a gaussian distribution.

§ Generation of Th1-cell lines

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

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

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

§ Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.00 and MedCalc for Windows. All data were expressed as mean ± standard deviation. Values of $p < 0.05$ were considered statistically significant.

In general, the significance of differences was evaluated by Wilcoxon rank sum test for unpaired data and student's two tailed *t*-test. The correlations were obtained using the Pearson's correlation. Inter-rater agreement was calculated through Weighted Kappa-coefficient. To assess the reliability of SARA score we also evaluated intra-class correlation coefficient (ICC).

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SUMMARY

Primary immunodeficiencies comprise more than 200 different disorders that affect the development and the functions of the immune system. Many scientific papers have been published on the molecular and cellular basis of the immune response and on the mechanisms involved in the correct development of immune system components. Although today we know the genetic and molecular basis of those principal mechanisms involved in the immune response, some aspect in this field remain unclear.

In this thesis, during the three years of my PhD program, I have contributed to elucidate “New insights and unsolved issues in congenital immunodeficiencies”, through the combination of clinical, cellular, functional and molecular approaches.

In particular, my research work is focused on the study role of γc in cell cycle progression, strongly related to its cellular amount and GH-R signaling, defining the basis of the physiological interaction between endocrine and immune systems. I demonstrate, moreover, that this subunit is able to influence the cell cycle progression in a concentration dependent manner in lymploblastoid and neoplastic cell lines.

Moreover, I participated to better define the functional role of FOXN1 transcription factor in the development of the T-cell ontogeny in the Nude/SCID syndrome. I also documented that FOXN1 is a possible cofactor in the development and differentiation of some structures in the central nervous system. Of note, this immunodeficiency is due to mutated gene expressed in non hematopoietic cells.

In addition, I participated to the study of patients affected with A-T. In this context I contributed to evaluate the beneficial effect of betamethasone on therapy in these patients. Our data indicate that betamethasone is effective in A-T at a minimal dosage and that GILZ may be a useful biomarker of the clinical response.

Finally, I also participate to give a contribution to the description of new mechanism in immunodeficiencies associated with unidentified molecular defects. Our data revealed that in a group of patients with high levels of serum IgE the IL-12R signaling was altered. Moreover, we documented the possibility of an autoreactive anti-lymphocyte antibody able to induce a SCID phenocopy.

Overall, all my studies were designed in order to clarify unsolved issues and unknown mechanisms underlying the functionality of the immune system. These results could be useful both in the clinical practice and in the basic research of immunedeficiencies.

ABBREVIATIONS

ADA = adenosine deaminase
APC = antigen presenting cells
AK2 = adenylate kinase 2
ALL = acute lymphoblastic leukemia
A-T = ataxia-telangiectasia
ATM = ataxia telangiectasia mutated
AZA = azathioprine
BCL = b cell line
BMP = bone morphogenetic protein
BMT = bone marrow transplantation
CNS = central nervous system
CNTF = ciliary neurotrophic factor
CSP = cavum septum pellucidum
DC = dyskeratosis ongenital
DN = double negative
DP = double positive
DSB = double-strand break
FOX = forkhead box
FGF = fibroblast growth factor
γc = common gamma chain
GC = glucocorticoid
GH = growth hormone
GHR = growth hormone receptor
GS-CSF = granulocyte-macrophage colony-stimulating factor
GILZ = glucocorticoid-induced leucine zipper
GRE = glucocorticoid responsive element
GVHD = graft-versus-host-disease
HIES = hyper ige syndrome
HPC = hematopoietic precursor cell
IGF = insulin growth factor
IL = interleukin
IRS = insulin receptor substrate
ISP = immature single positive
JAK = janus associated kinase
KGF = keratinocyte growth factor
LIF = leukemia inhibitory factor
MAPK = mitogen-activated protein kinase
MRI = magnetic resonance imaging

NK = natural killer
OSM = oncostatin m
PBMC = peripheral blood mononuclear cell
PI3K = phosphatidyl inositol 3 kinase
PK = protein kinase
PTP = tyrosine-protein phosphatase
RAG = recombinase activating gene
ROS = reactive oxygen species
RTE = recent thymic emigrant
SARA = scale for the assessment and rating of ataxia
SCID = severe combined immunodeficiency
SHP = sh2 domain-containing protein-tyrosine phosphatase
siRNA = short interfering rna
SOCS = suppressors of cytokine signaling
SP = single positive
STAT = signal transducers and activators of transcription
TCR = t cell receptor
TEC = thymic epithelial cell
TGF = tumor growth factor
TNF = tumor necrosis factor
Wnt = wingless

CURRICULUM VITAE
Dr. ILARIA VIGLIANO

PERSONAL INFORMATION

Name	**Ilaria, VIGLIANO**
Address	Viale I. D'Addedda, 316 - 71100 Foggia - Italy
Telephone	+39-0881-662080/ +39-349-2930011/ +39-3935666983
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Nationality	Italian
Date of birth	August 16, 1983

EDUCATION AND TRAINING

• Dates	From October 2008 - to October 2011
• Name and type of organisation providing education and training	Unit of immunology, Department of Pediatric at the "Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
• Principal subjects/occupational skills covered	Ph.D. student at the Doctoral Course in "Human Reproduction, Development and Growth" with a research project focused on the study of oncogenic role of IL2RG gene in the lymproliferative events.

• Dates	From 2006 - to 2008
• Name and type of organisation providing education and training	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
• Principal subjects/occupational skills covered	Internship at the Unit of Immunology, Department of Pediatrics of the "Federico II" University of Naples, Naples, Italy, focused on the study of a previously unappreciated relationship between common gamma chain and growth hormone receptor.
• Title of qualification awarded	Bachelor in Medical Biotechnology (II level degree) at the "Federico II" University of Naples, with a thesis entitled: "*Linfoproliferazione e Terapia Genica della X-SCID: Studio del ruolo della catena γ nella progressione del cclo cellulare*". Vote: 110/110 e lode
• Level in national classification	ISCED 6

• Dates	From 2005 - to 2006
• Name and type of organisation providing education and training	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.

• Principal subjects/occupational skills covered	Internship at the Unit of Immunology, Department of Pediatrics of the "Federico II" University of Naples, Naples, Italy, focused on the study of a previously unappreciated relationship between common gamma chain and growth hormone receptor.
• Title of qualification awarded	Bachelor in Biotechnology for Healthcare (I level degree) at the "Federico II" University of Naples with a thesis entitled: "Studio di traslocazione intranucleare di fattori di trascrizione in soggetti SCID X-Linked mediante tecnica confocale". Vote: 104/110.
• Level in national classification	ISCED 6
• Dates	From October 2009 - to October 2010
• Name and type of organisation	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
• Principal occupational	Tutor and teacher in Medicine school - Academic Year 2009/2010

PERSONAL SKILLS AND COMPETENCES

MOTHER TONGUE	ITALIAN
OTHER LANGUAGES	
	ENGLISH
• Reading skills	Good B1
• Writing skills	Good C2
• Verbal skills	Basic B1

SOCIAL SKILLS AND COMPETENCES

Predisposition to work in a team of 5-6 persorns by collaborating each other in a friendly manner and capacity to interact with other collegues also in multicultural environmnets developed during my PhD period.

ORGANIZATIONAL SKILLS AND COMPETENCES

- Capacity to design a scientific project (application to grant proposals) including the economic budget.
- Capacity to administrate small budgets for the daily work in a small lab.
- Capacity to coordinate students in their practice in lab also by follow them in the preparation of the thesis.

TECHNICAL SKILLS AND COMPETENCES

The competences were acquired during my PhD at the Department of Peditriatrics, where I also participated to clinical partice by diagnosting some immune disorders.

- Ability to perform a proliferative assay through the evaluation of thymidine incorporation by lymphocytes pre-stimulated with mitogens for diagnosis of the immunodeficiencies.
- Use of endnote 7.0 to format and add references to a manuscript.
- Use of Word, graphical softwares such as PowerPoint, Publisher, Photoshop, statitistical softwares such as Excel e GraphPad Prism and

softwares to elaborate images such as ImageJ 1.42.

- Knowledge of entrezgene, genecards, embl nucleotide sequence database and of the UCSC genome browser.

I also participated to the preparation of an entire scientific paper also by creating imagines and graphics and to the preparation of lessons, seminars and posters for congress and meetings.

OTHER SKILLS AND COMPETENCES

SCIENTIFIC SKILLS

- Cell cultures
- Separatione with $CD34^+$ microbeads (MACS)
- Set up of scaffolds
- DNA and RNA extractions (cells and tissues)
- PCR and Sequencing analysis
- Reverse transcriptase and Real-time PCR
- Transfection and RNA interference with lipofectamine
- Proliferation assay (thymidine, CFSE, MTS)
- Death assay (Trypan blue, Annexin V, Pridium Iodure)
- Western blot
- Immunofluorescence

ADDITIONALNAL INFORMATION ION

SCIENTIFIC INTERESTS

Major fields of my scientific interests are as follows:
- Regulatory mechanisms governing lymphocyte cell proliferation, activation and cell death. In particular, the study role of γc in cell cycle progression, strongly related to its cellular amount;
- Novel aspects in immunodeficiencies, with a particular regard to Severe Combined immunodeficiency (SCID) and molecular analysis of genes whose mutations are responsible for certain immunodeficiencies;
- The functional role of FOXN1 transcription factor in the development of the T-cell ontogeny in the Nude/SCID syndrome.
- Studies of previously unappreciated relationships between receptor signaling systems in the pathogenesis of SCIDs and signal transduction in physiology and human diseases affecting the immune system;
- Primary Immunodeficiencies: definition of novel therapeutical strategies for the treatment of Ataxia-Teleangiectasia. In particular, I participated in the activity of Prof. Pignata group during a clinical trial based on the use of bethametasone to improve neurological function in the affected patients

LIST OF PUBLICATIONS

- Amorosi S., Russo I., Amodio G., Garbi C., Vitello 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 182: 3304-3309, 2009.
- Pignata C., Fusco A., Amorosi S., **Vigliano I.**, Genovese V., Aloj G., Valentino L. La sindrome Nude/SCID: dal modello murino al fenotipo umano. RIGIP anno I numero 2, 2009.
- Amorosi S., Gorrese M., Fusco A., Vitiello L., Panico L., **Vigliano I.**, Ursini M. V, Racioppi L., Del Vecchio L., Pignata C. FOXN1 mutation abrogates pre-natal T-cell development in humans. Clin Immunol. 135: 315, 2010. (Abstract)
- Broccoletti T., Del Giudice E., Cirillo E., Giardino G., **Vigliano I.**, Ginocchio V. M., Bruscoli S., Riccardi C., Pignata C. Estimate the minimum therpeutically effective dosage of short-term therapy with Betamethasone on neurologicaal symptoms in patients affected with Ataxia-Telangectasia. Clin Immunol. 135: 314, 2010. (Abstract)
- Fusco A., Panico L., Troncone G., Amorosi S., **Vigliano I.**, Valentino L., Pignata C. Identification of extrathymic foci of lymphopoiesis in an athymic Nude/SCID human fetus. Clin Immunol. 135: 325, 2010. (Abstract)
- **Vigliano I.**, Amorosi S., Fusco A., Vitiello L., Palamaro L., Maio F:, Gallo V., Pignata C. Gamma chain expression level influences spontaneous cell proliferation in different malignant hematopoietic cell lines. Clin Immunol. 135: 326, 2010. (Abstract)
- 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. Cellular Immunology 265: 74-79, 2010.
- Broccoletti T., Del Giudice E., Cirillo E., **Vigliano I.**, Giardino G., Ginocchio V. M., Bruscoli S., Riccardi C. and Pignata C. Efficacy of very-low-dose betamethasone on neurological symptoms in ataxia-telangiectasia. European Journal of Neurology, 18: 564-570, 2011
- Amorosi S.[1], **Vigliano I.**[1], Del Giudice E., Panico L., Maruotti G. M., Fusco A., Quarantelli M., Ciccone C., Ursini M.V., Martinelli P., Pignata C. Brain alteration in a Nude/SCID fetus carrying FOXN1 homozygous mutation. Journal of the Neurological Sciences, 298:121-123, 2010 ([1]Contributed equally to this manuscript)
- Zanzi D., Stefanile, R., Santagata S., Iaffaldano L., Iaquinto G., Giardullo N., Lania G., **Vigliano I.**, Rotondi Vera A., Ferrara K., Auricchio S., Troncone R., Mozzarella G. IL-15 interferes with suppressive activity of intestinal regulatory T cells expanded in celiac disease. American Journal of Gastroenterology, 106:1308-1317, 2011

- **Vigliano I.**, Fusco A., Palalamaro L., Aloj G., Cirillo E., Salerno M.C., Pignata C. Gamma chain transducing element: a shared pathway between endocrine and immune system. Cellular Immunology 269: 10-15, 2011
- **Vigliano I.**, Gorrese M., Fusco A., Vitiello L., Amorosi S., Panico L., Ursini M.V., Calcagno G., Racioppi L., Del Vecchio L., Pignata C. FOXN1 mutation abrogates prenatal T-cell development in humans. J Medical Genetics, 48:413-416, 2011
- Palamaro L., Guarino V., Scalia G., Antonimi D., DeFalco L., **Vigliano I.**, Fusco A., Vitiello L., Giardino G., Caterina M., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T-lineage-committed cells. J Clin Immunol, 31 (Suppl 1): S49, 2011 (oral presentation)
- **Vigliano I.**, Fusco A., Panico L., Garrese M., Palamaro L., Bianchino G., Del Vecchio L., Pignata C. Potential extrathymic lymphopoiesis in a athymic human fetus carrying homozygous FOXN1 mutation. J Clin Immunol, 31(Suppl 1): S48, 2011 (oral presentation)
- 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 (in press)

AWARDS

- Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., Palamaro L., **Vigliano I.**, Pignata C. γ-chain provides a spontaneous and GH dependent sgnal fo cell cycle progression related to its cellular amount. Annual Meeting for the Federation of Clinical Immunology Societies. Boston 5-9 June, 2008 (poster)
- Amorosi S., Gorrese M., Fusco A., Vitiello L., Panico L., **Vigliano I.**, Ursini M. V, Racioppi L., Del Vecchio L., Pignata C. FOXN1 mutation abrogates pre-natal T-cell development in humans. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 May, 2010 (poster)
- Broccoletti T., Del Giudice E., Cirillo E., Giardino G., **Vigliano I.**, Ginocchio V. M., Bruscoli S., Riccardi C., Pignata C. Estimate the minimum therpeutically effective dosage of short-term therapy with Betamethasone on neurologicaal symptoms in patients affected with Ataxia-Telangectasia. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 May, 2010 (poster)
- Fusco A., Panico L., Troncone G., Amorosi S., **Vigliano I.**, Valentino L., Pignata C. Identification of extrathymic foci of lymphopoiesis in an athymic Nude/SCID human

fetus. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 May, 2010 (poster)

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

- **Vigliano I.**, Fusco A., Panico L., Garrese M., Palamaro L., Bianchino G., Del Vecchio L., Pignata C. Potential extrathymic lymphopoiesis in a athymic human fetus carrying homozygous FOXN1 mutation. Advanced School in Primary ImmunoDeficiency Disease Chicago 18-19 May, 2011 (oral presentation)

- Palamaro L., Guarino V., Scalia G., Antonimi D., DeFalco L., **Vigliano I.**, Fusco A., Vitiello L., Giardino G., Caterina M., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T-lineage-committed cells. Advanced School in Primary ImmunoDeficiency Disease Chicago 18-19 May 2011 (oral presentation)

GRANT PROPOSALS

- Bando Firb 2008 (MIUR) - Programma "Futuro in Ricerca", con un progetto dal titolo: **"**Characterization of the T-cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes";
- Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Bando MIUR 2008, con un progetto dal titolo: "Studio delle cellule T regolatorie e delle cellule Th17 nelle immunodeficienze primitive con autoimmunità".
- "TELETHON GRANT PROPOSALS-Call for Applications 2009" con un progetto dal titolo: "Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes";
- Bando Malattie Rare 2009 (Ministero della Salute) con un progetto dal titolo: "Potential oncogenic role of the x-scid gamma chain gene";
- Bando per la partecipazione alla selezione ai progetti di Ricerca Scientifica Finanziabili ai sensi della L.R. N.5 del 28.03.2002, con un progetto dal titolo: "Realizzazione di uno "scaffold" tridimensionale di policaprolattone per la generazione in vitro di linfociti T maturi a partire da cellule staminali".
- Fondazione Cariplo - Bando Ricerca Scientifica in ambito biomedico 2009, con un progetto dal titolo: "Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood".

- Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Bando MIUR 2009, con un progetto dal titolo: “Studio dell'ontogenesi delle cellule T in modelli umani di atimia ed autoimmunità: Allestimento di "scaffold" tridimensionali per la generazione in vitro di cellule T e Treg da precursori ematopoietici.”
- Fondazione Cariplo - Bando Ricerca Scientifica in ambito biomedico 2010, con un progetto dal titolo: “Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood”.
- “TELETHON GRANT PROPOSALS-Call for Applications 2010” con un progetto dal titolo: “Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes”.
- AIRC Application 2010, Potential oncogenic role of the X-SCID gamma chain gene.
- SIRPED – Società Italiana di Ricerca Pediatrica 2011, Modulation of molecular mechanism implicated in the high predisposition to infection and cancer in children with Ataxia-Teleangiectasia.