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PhD Thesis

Novel Aspects in Immunodeficiencies

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To my Brother

Index

Background and Aim of the Project

Primary immunodeficiency (PID) diseases are heritable disorders of the immune system (1). The field of the clinical description, genetic characterization, and immunological investigation of novel PIDs was born in the 1950s, but remains in its infancy, with considerable potential for growth (2). One can even predict that most specialties in human medicine will benefit considerably from the expansion of PIDs, as immunity at large is central to physiology and pathology, because most tissues are subject to environmental assaults and contain myeloid and lymphoid cells (2).

In this context my PhD program has been focused to the study of some of these diseases in order to clarify "*Novel Aspects in Immunodeficiencies*".

Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness. Apart from physical barriers, the immune response is composed from a diverse network of defenses, including cellular components and soluble mediators. A proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. The innate immune response involves three major cell types: phagocytic cells, such as neutrophils and macrophages, natural killer (NK) cells and antigen presenting cells (APC), which are also involved in the induction of an adaptive immune response. The adaptive immune system includes T and B lymphocytes responsible for cellular and humoral responses, respectively. However, these components of the immune system act in a well orchestrated and integrated unique system in order to maintain a normal resistance to infections.

In the last 5 decades, since the first human genetic defect was identified more than 200 PID syndromes have been described. PIDs can be divided into subgroups based on the component of the immune system that is predominantly affected, including T, B, NK lymphocytes, phagocytic cells and complement proteins. The antibody deficiencies (B‐cell or humoral immunodeficiencies) are characterized by genetic lesion, that selectively affects antibody production, but a normal cell-mediated immunity. In the cellular deficiencies, cellular effector mechanisms are compromised, whereas antibody production is largely normal in that B‐cell intrinsic machinery is intact. The combined immunodeficiencies are characterized by an impairment of both effector arms of the specific immunity, which results in a more severe clinical phenotype. However, since an efficient B-cell antibody response also depends on T-cell activation of B lymphocytes, defects in either cell type have the potential to affect both cellular and humoral immunity to varying degrees. Of note, most of the diseases within the last category are due to genetically determined blocks in the T-lymphocyte differentiation program. In the absence of mature T-cells, adaptive immunity is abrogated, thus resulting in a broad‐spectrum susceptibility to multiple pathogens also including opportunistic microorganisms. Overall, irrespectively of the pathogenic mechanism of the individual form of severe combined immunodeficiencies (SCIDs), a common hallmark of these diseases is the feature that bacterial, viral and fungal infections are often overwhelming. The discovery of a so wide number of distinct clinical entities which differ in either the genetic cause or the altered immunological function led to an incomparable increase in the knowledge of the intimate mechanism by which

a proper immune response is generated. Intriguingly, most of the genes whose alterations underlie PIDs are selectively expressed in hematopoietic precursor cells (HPC) with a few exceptions as, for example, Ataxia Telangiectasia Mutated (ATM) gene, also expressed in Purkinje cells and Adenosine Deaminase (ADA) which is ubiquitous. This dogma, however, led to underestimate those novel immunodeficiencies, which have different features involving other nonhematopoietic tissues.

This thesis reports the results I obtained during my PhD course in "Human Reproduction, Development and Growth" (XXII Cycle) from 2006 to 2009. During the past 3 years, my research has been focused in the study of the following 5 lines of research:

- Role of common gamma chain (gc) (X-SCID causing gene) in Growth Hormone Receptor signaling defining the basis of the physiological interaction between Endocrine and Immune Systems;
- Intrinsic property of gc as a key molecule in the cell cycle progression, spontaneous or GH-induced, its role being strongly related to its cellular amount;
- Molecular and clinical characterization of the human Nude/SCID phenotype and identification of clinical signs suggesting a previously unappreciated functional role of FOXN1 transcription factor in the development and differentiation of the central nervous system;
- Effects of steroid treatment in patients affected with Ataxia telangiectasia and the role of oxidative stress in its beneficial consequence;
- New insights in the T-cell ontogeny defects, studying different mechanisms or molecular alterations influencing both positive and negative selection processes.

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CHAPTER 1

The X-linked Severe Combined Immunodeficiency

Severe Combined Immunodeficiencies (SCIDs) represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T, B and NK cells (1). Most forms of SCID are associated with molecular alterations of genes selectively expressed in hematopoietic cells and implicated in cell differentiation/activation process. Mutations in nine different genes have been found to cause the human SCIDs.

The products of three genes, such as interleukin-2 receptor gamma (IL2RG), Janus associated kinase 3 (JAK3), and IL7R are components of cytokine receptors, while the products of RAG1, RAG2 and ARTEMIS are essential for antigen receptor gene rearrangement.

The most common form of SCID accounting for approximately half of all cases, with an estimated incidence of 1:150000 to 1:200000 live births is the X-linked severe combined immunodeficiency (X-SCID) (2-3).

This form of SCID is characterized by the absence of mature T and NK lymphocytes and absent immunoglobulin synthesis, despite the presence of a normal or sometime elevated number of B cells. In X-SCID patients, T cells are absent not only in the peripheral blood but also in the central and peripheral lymphoid organs suggesting an early block in the T-cell differentiation pathway (4). Furthermore, although peripheral B cells exhibit a normal phenotype, X-SCID patients B cells are not functional, even after T-cell reconstitution by means of bone marrow transplantation (5-6).

§ 1.1 X-linked Severe Combined Immunodeficiency: the gamma chain transducing element

The most common form of SCID is the X-linked form that accounts for 40–50% of all cases. This form is frequently caused by alterations of the *IL2RG* gene that encodes for the common cytokine receptor gc, a shared component of several cytokine receptors.

A wide range of cytokines have been molecularly recognized. They are soluble elements that control the immune and the hematopoietic system (7). Their pleiotropic and redundant functions are due to the various receptors expressed on multiple target cells, so, cytokines' specific rules are closely dependent on the recognized targets. Therefore, for appreciating such cytokine functions, a lot of cytokine receptors have also been molecularly studied and characterized. These are classified into five families, created on the bases of extra- and intra-cellular domains structure affinity: the cytokine receptor superfamily, interferon receptor family, TNF (Tumor Necrosis Factor) receptor family, TGF (Tumor Growth Factor)-b receptor family, and IL-8 (Interleukin-8) receptor family (8). The cytokine receptor superfamily is the largest family, containing no less than 18 different receptor molecules, a quantity of which may be included among multiple cytokine receptors; the receptors for IL-6, IL-11, OSM, CNTF, and LIF contain the common gp130, and the receptors for IL-3, IL-5, and GM-CSF enclose the common gc. Essentially, gc and gp130 contribute to increase the ligand-binding affinity and to establish an intracellular signal transduction (8-9).

The gc is a component of several receptors such as for IL-2 receptor (IL-2R), IL-4R, IL-7R, IL-9R and IL-15R. To our knowledge, an extrahemopoietic role of gc has not yet been demonstrated, although the abundance of the protein in nonhemopoietic cells would imply additional functions for this element (10-11).

The signals, that are dependent on gc, have been shown to be important for various immunological function. The characterization of cytokine-activated genes, including genes regulated by gc-dependent cytokines, has long been an area of considerable interest, although only few systematic studies have been conducted. These studies have now allowed the identification of gene expression programs involved in complex biological processes including studies regarding genes induced by gc-dependent cytokines (12-13) and of changes in gene expression that take place during T-cell differentiation either in Th1 or Th2 conditions (14).

Even though the role of different cytokine-dependent signaling pathways in the regulation of gene responses is still unresolved, there are a wide variety of evidences that the common cytokine receptors gc cytokines control the immune response at different as well as overlapping checkpoints, and their expression levels are diversely controlled during an immune response (Table 2). In fact, IL-2, IL-7 and IL-15 were reported to regulate a highly overlapping set of more than one hundred genes, exerting diverse effects correlated to T-cell survival, activation and clonal expansion, and to the development and preservation of cell memory. Moreover, IL-7 regulates lymphocyte development and homeostasis. Furthermore, an important role for IL-7 in T- and B-cell development was first suggested by experiments in which B- and T-cell development was abrogated when mice were injected

with antibodies to either IL-7 (15-17). In fact, IL-7 has an action based on an increase of cell survival correlated to an induction of *Bcl-2* gene expression. However, other cytokines such as IL-15 and IL-2 may be more important. In fact, although IL-7R is highly expressed on resting T cells, it is rapidly downregulated by T cell Receptor (TCR) stimulation as well as by IL-2, further supporting the idea that other cytokines are more relevant for effector functions (18). In particular, both IL-2 and IL-15 are T-cell growth factors *in vitro,* and they can stimulate the proliferation of NK cells as well as induce NK cell cytolytic activity. However, important *in vivo* differences in the actions of these two cytokines have emerged, particularly, in regard to NK cell development and CD8b T-cell homeostasis (19). As noted, IL-2Ra deficient mice show generally normal T, B and NK cell development. However, Il-2rb-deficient mice have profoundly decreased numbers of NK cells and g/d T cells, suggesting that IL-15 but not IL-2 is necessary for the development/differentiation of these cells. Indeed, mice deficient in either Il-15 or Il-15ra lack NK cells, confirming the distinctive role for IL-15 in NK cell development (20-21). Thus, IL-15 is essential for the homeostatic proliferation of memory CD8+ T cells and maintenance of the steady-state level of CD8+ T-cell memory, exerting, furthermore, direct effects on memory CD8+ T cells and on other cell types, which subsequently control memory T-cell proliferation. On the other hand, IL-9 is a mast cell growth factor and was first identified as a late-acting T-cell growth factor and mast cell growth factor. Il-9-deficient mice have also been generated, and the lymphoid compartment develops normally in these animals. However, these mice exhibit excessive mucus production and mast cell proliferation (22). But, such abnormalities have not been reported in humans with X-SCID,

suggesting that the gc isn't responsible of this phenomenon. Interestingly, Il-9 transgenic mice develop thymic lymphomas, consistent with the presence of Il-9 receptors in the thymus and with the ability of thymocytes to respond to Il-9. Instead, roles of IL-4 and IL-21 in Th-cell differentiation and immunoglobulin (Ig) Synthesis Gene knockout models of different gc-dependent cytokines have revealed roles for IL-4 and IL-21 in the regulation of Ig production. Early work had indicated a role for IL-4 in B-cell Ig class-switch to IgG1 and IgE (23).

It is now clear that gc 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 gc-dependent cytokines is of central importance. Thus, abnormality of either one of them can have a profound impact on the homeostasis of the immune system. Undoubtedly, a balanced act of gc-cytokines is critically important in this regard. However, given the widespread use of gc among cytokine receptors, this pathway is likely to have implications for anti-apoptotic signaling in a variety of contexts (24).

Ursini et al. in 2002 reported on a patient affected with X-SCID, due to gc alteration, who received a bone marrow transplantation late at 5.2 years of age (25). In this patient, short stature became evident and a peripheral growth hormone (GH) hypo responsiveness associated with abnormalities of the protein phosphorylation events that occur following GH receptor (GHR) stimulation was demonstrated. After this observation, since that the abundance of gc in non hematopoietic tissues was largely demonstrated, we

hypothized that the GH hypo responsiveness was due to the hematopoietic alteration.

The potential role of gc in GHR signalling was assessed using B cell lines (BCLs) from healthy control and gc-negative X-SCID patients. In particular, the authors demonstrated the impairment of gc in various GH-induced events. At first, the role of gc became evident, by proliferation assay, in the functional response of both kinds of cells to GH in a dose-dependent manner. In fact, GH enhanced proliferation of control BCLs in a dose-dependent fashion. By contrast, the functional response to GH of the BCLs of gc-negative patients was severely impaired despite a comparable cellular expression of GHR molecules.

Furthermore, the signal transduction properties of GHR in X-SCID patients and control BCLs following GH stimulation was also examined by analyzing the pattern of protein tyrosine phosphorylation. The activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (26). Other signaling pathways also contribute to a full GHR response, in fact, GH has been shown to activate the PI3K protein kinase B signaling (27), MAPKs, and ERKs 1 and 2 (28-30). In contrast to what observed in control BCLs, in X-SCID patients GH stimulation failed to induce phosphorylation of one of the STAT family molecules, involved in the signal transduction through GHR. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in the cell lines of gc-negative patients in contrast to the control cells, in which a rapid activation of STAT5 occurred. To define whether the blockage in GHR signaling was specific of STAT5 or involved other molecules as well, JAK2, STAT1, ERKs, and STAT3 phosphorylation were studied. No difference in

the phosphorylation events between the BCLs of patients and controls was appreciable and the expression of the molecules was comparable in control and patient cells.

Moreover, as a strongly support of the interaction between GHR and gc, the reconstitution of X-SCID cells with the wild-type gc gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5, instead functional silencing of gc, obtained using a neutralizing Mab, causes a decrease in cellular response to GH.

So, these data suggest that the gc is a necessary subunit of the GHR signaling complex in BCLs, where it is required for STAT5 phosphorylation and nuclear translocation, and not for the activation of other molecules of GHR signalling apparatus.

These data have been published on *The Journal of Immunology* and reviewed on *Current Signal Transduction Therapy*, for the manuscripts see below.

Functional Interaction of Common γ-Chain and Growth Hormone Receptor Signaling Apparatus[1]

Marsilio Adriani,* Corrado Garbi,† Giada Amodio,* Ilaria Russo,* Marica Giovannini,* Stefania Amorosi,* Eliana Matrecano,* Elena Cosentini,‡ Fabio Candotti,§ and Claudio Pignata[2]*

We previously reported on an X-linked SCID (X-SCID) patient, who also had peripheral growth hormone (GH) hyporesponsiveness and abnormalities of the protein phosphorylation events following GH receptor (GHR) stimulation. In the present study, we examined a potential role of common cytokine receptor γ-chain (γ_c) in GHR signaling using EBV-transformed lymphocytes from healthy subjects and γ_c-negative X-SCID patients. We demonstrated that the proliferative response to GH stimulation of the B cell lines of γ_c-negative patients was impaired despite a comparable cellular expression of GHR molecules to controls. In patients, after GH stimulation, no phosphorylation of STAT5 was observed. In addition, the molecule localization through confocal microscopy revealed that in B cell lines of patients no nuclear translocation of STAT5b following GH stimulation occurred differently from controls. Biochemical analysis of the nuclear extracts of γ_c-negative cell lines provided further evidence that the amount of STAT5b and its phosphorylated form did not increase following GH stimulation. In patients, cells reconstituted with wild-type γ_c abnormal biochemical and functional events were restored resulting in nuclear translocation of STAT5. Confocal experiments revealed that GHR and γ_c were colocalized on the cell membrane. Our study demonstrates the existence of a previously unappreciated relationship between GHR-signaling pathway and γ_c, which is required for the activation of STAT5b in B cell lines. These data also confirm that growth failure in X-SCID is primarily related to the genetic alteration of the *IL2RG* gene. *The Journal of Immunology,* 2006, 177: 6889–6895.

Severe combined immunodeficiencies represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T, B, and NK cell (1). Most forms of SCID are associated with molecular alterations of genes selectively expressed in hemopoietic cells and implicated in the cell differentiation/activation process. Thus, classical symptoms are generally considered those related to the immunological impairment that results in increased susceptibility to infections. Because patients usually die by the first year of age without an effective treatment, the clinical phenotype is predominated by the life-threatening problems.

X-linked SCID (X-SCID)[3] is the most common form of the disease accounting for approximately half of all cases (2, 3). The gene responsible for X-SCID is *IL2RG* that encodes for the common cytokine receptor γ-chain (γ_c), a member of the cytokine receptor class 1 superfamily. The molecule represents a shared component of several receptors critical for the development and function of lymphocytes (3). To our knowledge, an extrahemopoietic role of γ_c has not yet been demonstrated, although the abundance of the protein in nonhemopoietic cells would imply additional functions for this element (4, 5).

We previously reported on a patient affected with X-SCID who received a bone marrow transplantation late at 5.2 years of age. In this patient, short stature became evident, and a peripheral growth hormone (GH) hyporesponsiveness associated with abnormalities of the protein phosphorylation events that occur following GH receptor (GHR) stimulation was demonstrated.

The GHR was the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for erythropoietin, G-CSF, GM-CSF, IL-2–7, IL-9, IL-11, IL-12, and many other cytokines. Due to the lack of intrinsic kinase activity, members of the cytokine receptor superfamily recruit and/or activate cytoplasmic tyrosine kinases to relay their cellular signal. The JAK2 represents the predominant nonreceptor tyrosine kinase required for the initiation of GH signal transduction upon ligand binding to the receptor (6, 7). However, GH also stimulates tyrosine phosphorylation of JAK1 (8, 9) and JAK3 (10) in certain cell lines. Signal transduction through GHR also involves a wide array of molecules, such as STATs 1, 3, and 5, ERK 1 and 2, and PI3K-protein kinase B (7). Activation of STAT5b is considered a prominent event in GH signaling and is crucial for the regulation of transcription of GH-responsive genes, including the gene encoding for insulin-like growth factor (IGF)-I, which mediates many of the GH biological functions (11–13). In our previous study, mutational screening and expressional analysis failed to reveal any molecular alteration of *GHR*, *JAK2*, and *STAT5A/B* genes in the patient with X-SCID and peripheral GH hyporesponsiveness (14).

Because we hypothesized a role for the γ_c in GHR signaling, in this study, we evaluate the functional interaction between GHR

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[3] Abbreviations used in this paper: X-SCID, X-linked SCID; γ_c, common cytokine receptor γ-chain; GH, growth hormone; GHR, GH receptor; IGF, insulin-like growth factor; rGH, recombinant human GH; BCL, lymphoblastoid cell line; WT, wild type.

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and the common γ element in either freshly isolated or EBV-transformed lymphocytes from X-SCID patients and healthy subjects. In particular, the functional response to GH stimulation, the pattern of GHR-induced protein tyrosine phosphorylation and GH-induced translocation from the cytoplasm to the nucleus of STAT5 were evaluated. We demonstrate the existence of a previously unappreciated functional interaction between γ_c and GHR. This interaction leads to the activation and intranuclear translocation of the STAT5b protein.

Materials and Methods

Reagents

Recombinant human GH (rGH) was obtained from Serono (Saizer 4). The ECL kit was purchased from Amersham Biosciences. The Abs anti-STAT5b, anti-STAT5a, anti-STAT1, anti-STAT3, anti-ERK (recognizing both ERK1 and ERK2), anti-phosphotyrosine ERK, anti-GHR and anti-γ_c and the mAbs anti-phosphotyrosine were purchased from Santa Cruz Biotechnology. The Ab anti-JAK2 was purchased from Cell Signaling Technology. The neutralizing IgG1 anti-γ_c mAb was purchased from R&D Systems. An IgG1 isotype-matched anti-CD3 mAb (Leu 3, UCHT1 clone) was purchased from BD Biosciences. Epidermal growth factor was purchased from BD Biosciences and used at the concentration of 100 ng/ml. Acrylamide and bisacrylamide were obtained from Invitrogen Life Technologies. Prestained molecular mass standards were obtained from Bio-Rad. Except where noted, other reagents were of either reagent or molecular biological grade from Sigma-Aldrich.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from four X-SCID patients and normal donors and heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by EBV immortalization of patients and control PBMC using standard procedures (15). In all cases, γ_c mutations led to the absence of protein expression. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM/L L-glutamine (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Invitrogen Life Technologies), and cultured at 37°C, 5% CO_2. In BCL transduction experiments, the pGC2Rγ retroviral vector (16) was used to transduce X-SCID BCLs with wild-type (WT) γ_c as previously described (17). Transduced cells were selected in the neomycin-analog G418 (Cellgro). NIH 3T3 fibroblasts were used in a few experiments.

Proliferative assay

BCLs (1×10^5 cell/200 μl well) were cultured triplicate in 96-well U-bottom microtiter plates (Falcon; BD Biosciences) with or without rGH at reported concentrations for 4 days. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences) 8 h before harvesting (18). In neutralization experiments, control EBV cells were preincubated with the neutralizing mAb 284 at the concentration of 6 ng/ml for 3 h or with the IgG1 isotype-matched Ab (Leu 3).

Flow cytometry

The expression of GHR was detected using specific rabbit Abs (Santa Cruz Biotechnology) by indirect immunofluorescence using a second-step incubation with FITC-conjugated donkey anti-rabbit Abs (Pierce). After washing in PBS, cells were incubated for 20 min with the specific Abs and 30 min with secondary Abs. After staining, all samples were washed in PBS and acquired on the FACScan flow cytometer (BD Biosciences) using Lysis I software.

Cell stimulation and protein extraction

Before hormone treatment, the cells were made quiescent through incubation in RPMI 1640 minus serum for 8–12 h. GH was used at 37°C at a concentration of 500 ng/ml in RPMI 1640 for the reported time. Incubations were terminated by washing cells with ice-cold PBS (BioWhittaker) followed by solubilization in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadatum (Na_3VO_4), 5 μg/ml leupeptin, and 5 μg/ml aprotinin. The cell lysates were stored at −80°C for Western blot analysis. Nuclear extracts were prepared by the method of Andrews et al. (19) and were subsequently mixed with sample buffer.

Western blot

Immunoblotting using phosphotyrosine mAb was performed as previously reported (14). Immunoblotting using specific Ab was performed according to the vendor protocols. In brief, protein samples separated by SDS-PAGE were transferred onto Mixed Cellulose Esters membranes (Immobilon-NC Mixed Cellulose Esters 0.45 μm; Millipore). The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 10% BSA in wash buffer (10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20). The membrane was then washed three times in wash buffer and incubated 1 h at room temperature or overnight at 4°C with the specific Ab. The membrane was then washed three times and an appropriate IgG HRP-conjugated secondary Ab was used for the second incubation. After further washings, the membrane was developed with ECL-developing reagents, and exposed to x-ray films according to the manufacturer's instructions (Amersham Biosciences).

Confocal microscopy

After appropriate stimulation, quiescent cells were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature. After four rinses of 5 min in PBS, the cells were 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. The cells were then incubated for 1 h at room temperature with rabbit Abs against STAT5b diluted 1/100 in PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with a 1/200 dilution of FITC-conjugated donkey anti-rabbit IgG (Pierce) in PBS. After washing in PBS, the glass slides were mounted under a coverslip in a 50% glycerol/50% PBS solution. The slides were analyzed by laser scanning confocal microscopy, using a Zeiss LSM 510 version 2.8 SP1 Confocal System.

Results

Effect of GH on the proliferative response of EBV-transformed cell lines from normal subjects and γ_c-negative X-SCID patients

It has been reported that GH enhances EBV-transformed cell line proliferation in vitro, its effect being direct and not mediated by IGF-I (18). Thus, to evaluate a biological role of γ_c in GHR signaling, we evaluated the response of EBV-transformed lymphocytes (BCLs) from γ_c-negative X-SCID patients and normal controls to GH stimulation. As shown in Fig. 1*A*, GH enhanced proliferation of BCLs of control subjects in a dose-dependent fashion. Significant enhancement of [^{3}H]thymidine uptake was observed at a GH concentration of 50 ng/ml, and the maximal effect was achieved at 200 ng/ml. In contrast, γ_c-negative BCLs did not respond at any GH concentration. To rule out that the observed phenomena were due to different numbers of the receptor molecules on the cell membrane, GHR expression was evaluated by flow cytometry analysis of the cells of controls and patients (Fig. 1*B*). No difference was found in the mean fluorescence intensity (130.99 ± 28.19 vs 139.88 ± 33.49 in patients and controls, respectively; p = NS) and in the percentage of positively stained cells (99.6 vs 99.7% and 99.8 vs 99.9%, respectively).

Moreover, to demonstrate a link between the γ_c and GHR, we used a neutralizing mAb in the proliferative assay. As shown in the Fig. 1*C*, the neutralizing mAb inhibited by 64% the proliferative response to GH. A nonspecific effect of the Ab was ruled out, because the IgG1 anti-CD3 isotype-matched Ab was ineffective in inhibiting cell proliferation.

To define whether PI3K had a role on GH-induced cell proliferation of BCLs to GH, the kinase inhibitor wortmannin was used. As shown in Fig. 1*D*, no inhibitory effect was appreciable. By contrast, in the positive control wortmannin was able to inhibit fibroblast proliferation to EGF by 85%.

To ascertain whether γ_c was linked to GHR, we then assessed by confocal microscopy the plasma membrane expression of these two molecules. As shown in Fig. 1*D*, by indirect immunofluorescence using specific Abs, as previously detailed, colocalization of γ_c and GHR was observed on the cell surface of normal BCL cells.

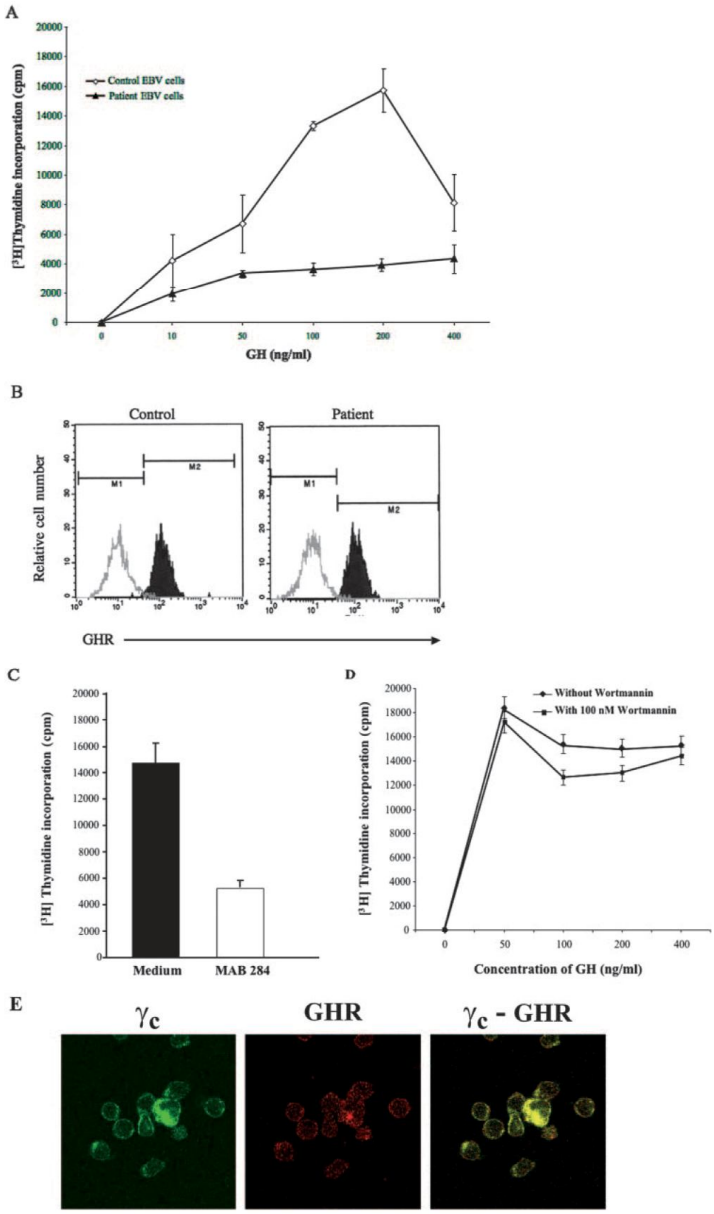

FIGURE 1. In vitro effect of GH stimulation on proliferation of the EBV cell lines of X-SCID patients and controls and membrane localization of GHR and γ_c. *A*, BCLs were generated by EBV immortalization of the PBMC of patients and controls using standard procedures (15) and cultured in the presence of various concentrations of GH for 4 days. Cultures were pulsed with [^{3}H]thymidine for the final 8 h and radioactive incorporation counted. Results are expressed as the increase of cpm from the background. Vertical bars indicate 1 SD. *B*, Flow cytometry analysis indicating that the expression levels of GHR are comparable in controls and patients. *C*, Control BCLs were pretreated with medium alone (■) or with the neutralizing mAb 284 (□) at the concentration of 6 ng/ml for 3 h, and then cultured for 4 days in the presence of GH at the concentration of 200 ng/ml. As isotype-matched IgG1 control Ab, anti-CD3 Leu 3 was used. Cultures were processed as previously described. *D*, Control BCLs were pretreated for 1 h with medium alone or with wortmannin at the concentration of 100 nM, and then stimulated with 50, 100, 200, or 400 ng/ml GH, as indicated. As a positive control, fibroblasts were cultured in the presence of EGF. *E*, γ-chain colocalizes with GHR. Normal BCL cells were double labeled with anti-γ_c (*left*) and anti-GHR (*center*) Abs. Confocal microscopic analysis indicates a plasma membrane localization for both molecules. The yellow color in the merge (shown on the *right*) indicates areas of colocalization of the two proteins.

Pattern of protein tyrosine phosphorylation induced through GHR engagement in patients and controls cells

We next investigated the overall signal transduction properties of patients and control BCLs following GHR ligation by analyzing the number and the timing of the proteins phosphorylated on tyrosine residues. Fig. 2 illustrates a representative immunoblot with anti-phosphotyrosine Abs of whole cell lysates from BCLs of patients and controls BCLs following stimulation with GH for 5, 15, or 30 min. In contrast to what was observed in control cells, in patients, GH stimulation failed to induce phosphorylation of

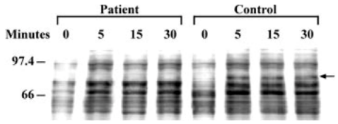

FIGURE 2. Pattern of protein tyrosine phosphorylation induced through GHR engagement. BCLs from X-SCID patients and healthy subjects were starved of serum for 8–12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. Stimulation was stopped with cold PBS and BCLs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-phosphotyrosine Abs.

proteins of ~90 kDa, presumably corresponding to STAT molecules involved in the signal transduction through GHR. This pattern of protein tyrosine phosphorylation was also observed in freshly isolated PBMC from a healthy subject and a patient stimulated with the same concentration and for the same time, thus confirming the observation on BCLs (data not shown).

GHR signal transduction pattern

The three main signal modules by which signal transduction through GHR occurs involve MAPK/ERK1 and 2, JAK2, STAT1, STAT3 and STAT5 and the PI3K-protein kinase B signaling (7).

To evaluate whether the γ_c was involved in GHR-signaling events, we first focused on STAT5 molecule. As shown in Fig. 3, in the BCLs of controls, tyrosine phosphorylation of STAT5 was evident, with a peak of activity observed between 5 and 15 min after GH stimulation. By contrast, in the BCLs of patients, no phosphorylation of STAT5 was detectable after stimulation. In all cell lines examined, STAT5b and STAT5a protein expression was comparable in patients and controls.

To define whether the blockage in GHR signaling was specific of STAT5 or involved other molecules as well, we then studied JAK2, STAT1, ERKs, and STAT3 phosphorylation (Fig. 4). No difference in the phosphorylation events between the BCLs of patients and controls was appreciable. As shown, the expression of the molecules was comparable in control and patient cells.

STAT5 nuclear translocation after GHR triggering

Recently, it has been reported that tyrosine phosphorylation of STATs molecules was not sufficient for the activation of the protein (20, 21). Because the activated STAT5 translocates into the nuclei, confocal microscopy was initially used to test the subcellular localization of STAT5b in control and patient γ_c-negative cells under resting conditions and after stimulation with GH.

BCLs of patients and controls were stimulated with GH for 30 min, fixed, and incubated with antiserum against STAT5b. As shown in Fig. 4, under basal conditions all cells displayed fluorescent staining of the cytoplasm indicating the presence of STAT5b in this compartment, and only a negligible staining of nucleus indicating absence of STAT5b in this compartment. Stimulation with GH for the time indicated induced nuclear translocation of STAT5b in the BCLs of controls, as demonstrated by the marked increase in STAT5b immunoreactivity within the nucleus and not in γ_c-negative BCLs.

We next evaluated by immunoblot of nuclear and cytoplasmic extracts the amount of STAT5b translocation and compared it with the tyrosine phosphorylation of the molecule. As shown in Fig. 5, in control cells, GH stimulation determined a rapid increase of nuclear STAT5b amount. The translocation occurred early being evident 5 min after GH stimulation. Moreover, it still persisted 30 min after stimulation. The translocation paralleled the amount of the tyrosine-phosphorylated form of the protein into the nuclei. This was inversely correlated with the amount of the cytoplasmic form of the molecule. However, after 30 min, the reconstitution of the cytoplasmic aliquot became evident. In the patient cells, no changes were observed (Fig. 6).

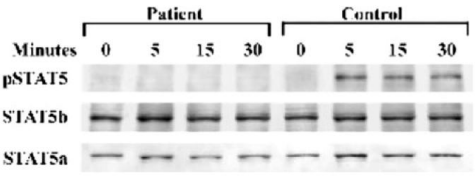

FIGURE 3. STAT5 phosphorylation induced through GHR stimulation. rGH stimulation failed to induce STAT5 tyrosine phosphorylation in γ_c-negative BCLs. BCLs from X-SCID patients and healthy subjects were starved of serum for 8–12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. After SDS-PAGE and Western blot, membranes were incubated with anti-pSTAT5, anti-STAT5b, or anti-STAT5a Abs.

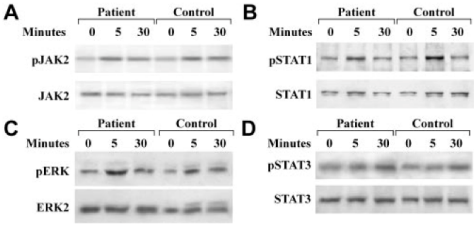

FIGURE 4. Phosphorylation events induced through GHR stimulation. BCLs from X-SCID patients and healthy subjects, starved of serum for 8–12 h, were stimulated for the indicated time with rGH at the concentration of 500 ng/ml. After SDS-PAGE and Western blot, membranes were incubated with (*A*) anti-pJAK2 or anti-JAK2, (*B*) anti-pSTAT1 or anti-STAT1, (*C*) anti-pERK or anti-ERK2, (*D*) anti-pSTAT3 or anti-STAT3.

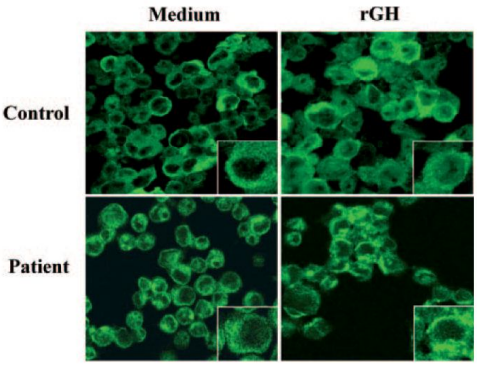

FIGURE 5. STAT5b subcellular localization. Control cells of X-SCID patients and healthy subjects were cultured in the absence or presence of 500 ng/ml rGH for 30 min at 37°C. Unstimulated or stimulated cells were analyzed by confocal microscopy for STAT5b (green) distribution in the cell, focusing particularly on whether this protein was present in the nuclei.

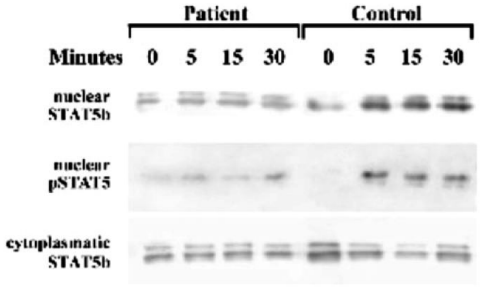

FIGURE 6. Nuclear fraction of the overall STAT5b amount and of the phosphorylated form of STAT5 in resting or rGH-stimulated BCLs. Patient and control BCLs were stimulated with rGH (500 ng/ml) or medium alone at 37°C for the indicated time. Stimulation was stopped with cold PBS and nuclei were isolated as described in *Materials and Methods*. After SDS-PAGE of nuclear and cytoplasmic extracts and Western blot, membranes were incubated with anti-STAT5b or anti-pSTAT5 Abs.

GH-induced signaling and STAT5b nuclear translocation in X-SCID EBV cells transduced with the WT γ_c gene

We next evaluated whether reconstitution of X-SCID cells with WT γ_c led to a functional recovery. As shown in Fig. 7*A*, pGC2Rγ cells expressed γ_c at a normal extent. These cells proliferated in a comparable fashion to control cells following GH stimulation (Fig. 7*B*). Moreover, in WB experiments using an anti-phospho-STAT5 Ab, a phosphorylation of the molecule was observed in pGC2Rγ cells (Fig. 7*C*). Finally, in reconstituted cells, GH stimulation induced a normal nuclear translocation of STAT5b, as shown in Fig. 7*D*.

Discussion

In the present study, we examined a potential role of the γ_c chain in GHR signaling using BCLs from healthy control subjects and γ_c-negative X-SCID patients. At a functional level, GH enhanced proliferation of control BCLs in a dose-dependent fashion. By contrast, the functional response to GH of the BCLs of γ_c-negative patients was severely impaired despite a comparable cellular expression of GHR molecules.

The overall signal transduction properties of GHR in X-SCID patients and control BCLs following GH stimulation was also examined by analyzing the pattern of protein tyrosine phosphorylation. In contrast to what was observed in control BCLs, in patients, GH stimulation failed to induce phosphorylation of proteins of 90 kDa identified as belonging to the STAT molecules family, involved in the signal transduction through GHR. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in the cell lines of γ_c-negative patients in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with the WT γ_c gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γ_c in GHR signaling.

STAT-dependent pathways are generally believed to be used in cellular events such as cell proliferation, differentiation, and apoptosis (22, 23), even though the overall role of the STAT molecules in GHR signal transduction has not been fully elucidated. At least three different STAT family members (STAT1, STAT3, and STAT5) are activated following GHR perturbation (24–29), even though STAT5 seems to play a prominent role in receptor signaling. Rodent models of STAT knockouts (30) and the recent identification of a patient with a homozygous missense mutation of the

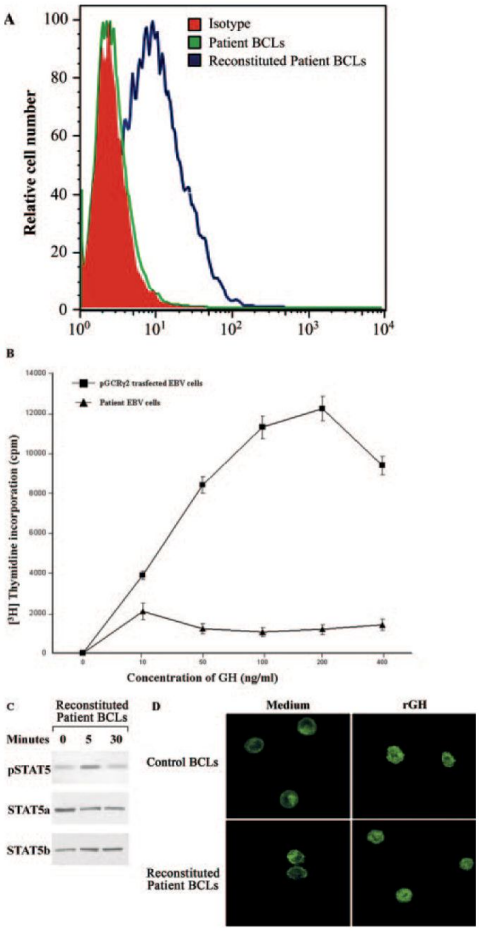

FIGURE 7. GHR signaling in patient BCLs reconstituted with WT γ_c (pGC2Rγ cells). *A*, Membrane expression of γ_c in patient or pGC2Rγ cells by flow cytometry. *B*, Proliferative response in control, patient, or reconstituted BCLs. Cells were cultured in the presence of various concentrations of GH for 4 days and pulsed with [^{3}H]thymidine as previously described. Results are expressed as increase of cpm from the background. Vertical bars indicate 1 SD. *C*, STAT5 phosphorylation induced through GHR stimulation for the indicated time in pGC2Rγ cells. Membranes were incubated, as indicated, with anti-pSTAT5, anti-STAT5a, or anti-STAT5b Abs. *D*, STAT5b subcellular localization through confocal microscopy analysis in control or pGC2Rγ cells unstimulated or stimulated with 500 ng/ml rGH for 30 min at 37°C.

STAT5b gene indicate that STAT5b is essential for a normal postnatal linear growth (31). Furthermore, the patient with STAT5b mutation also had clinical features of immune deficiency such as chronic diarrhea and severe infections, including interstitial pneumopathy. Immunologic studies showed hypergammaglobulinemia and markedly decreased IL-2Rα-chain expression in response to IL-2 stimulation, suggestive of a T cell activation defect. Thus, a few features are similar to γ_c-negative X-SCID patients.

Although the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (32), other signaling pathways also contribute to a full GHR response. GH has been shown to activate the PI3K-protein kinase B signaling (33), MAPKs, and ERKs 1 and 2 (34–36). In both STAT5 knockout mouse and in the patient with STAT5 mutation, these pathways are fully functional. In keeping with this observation, also in our experimental model, no alteration was observed in ERK 1 and 2 expression and phosphorylation events involving JAK2, ERKs, STAT1 and 3 molecules that occur following GHR triggering. Moreover, in this study, the involvement of PI3K in GH-induced proliferation of BCLs was ruled out because the kinase inhibitor wortmannin was ineffective in blocking the proliferative response. Similarly, IGF-I expression has been reported to be dependent on STAT5 b, but not on the PI3K pathway (37). Taken together, these observations imply that GHR, as well as other receptors, is able to integrate different pathways which are individually differentially regulated. In support of this, it has been recently shown that GHR signaling and the subsequent IGF-I transcription regulation are under different regulatory controls in hepatocytes, fibroblasts, and myoblasts (38). This could lead to a hypothesis of differential functions of an individual receptor exerted in different tissues. A cell type-restricted STAT activation has been reported (39–41). STAT5 is not activated following GH stimulation in human fibrosarcoma cells even though these cells express the STAT5 protein (41), thus implying that a selectivity in the involvement of specific STAT subset seems to be a general feature of GHR signal transduction.

Overall, activation of STAT5b is considered a prominent event in GHR signaling and is crucial for the regulation of transcription of GH-responsive genes, including the gene encoding for IGF-I. This process relies on an appropriate phosphorylation and nuclear translocation of the molecule (7, 42). Recently, it has been proposed that STATs tyrosine phosphorylation and nuclear translocation are two events that are regulated separately (21). In particular, Giron-Michel et al. (43) demonstrated in the hybrid receptor γ_c/GM-CSFRβ that the γ_c/JAK3 complex controls the nuclear translocation of pSTAT5 rather than STAT5 phosphorylation itself. Hence, to address the issue of defining the functional implication of γ_c mutation on STAT5b activation, in our study, the subcellular localization of STAT5b was investigated by analyzing cytokine-induced translocation of STAT5b from the cytoplasm to the nucleus with confocal microscopy. Stimulation with GH induced nuclear translocation of STAT5b in the control cells, whereas no efficient nuclear translocation occurred in γ_c-negative cells. Furthermore, immunoblot of nuclear and cytoplasmic extracts showed in control cells a rapid increase of the nuclear fraction of the STAT5 molecule after GH stimulation, which paralleled the molecule phosphorylation, differently from what was observed in patient cells. Moreover, through confocal microscopy studies, we demonstrated that GHR and γ_c colocalize, as expected in that both molecules are type I hemopoietin receptors. A physical interaction may be hypothesized as well, even though conclusive data are still lacking.

Our data suggest that the γ_c chain is a required signaling subunit of the GHR complex in B cell lines. In particular, in this cell line, it is selectively required for STAT5 phosphorylation and nuclear translocation, and not for the activation of other molecules as ERKs.

Our study demonstrates the existence of a previously unappreciated relationship between individually well-studied elements, such as GHR and γ_c, and signaling pathways. Cross-talk between receptor signaling systems is now emerging as an important and exciting area of signaling research. Whether the participation of γ_c to the GHR confers some additional properties to the receptor in hemopoietic cell differentiation and functioning remains to be elucidated. Of note, in CD34$^+$ progenitors, γ_c participates in hemopoietic cell differentiation by interacting with GM-CSFRβ. This interaction does not occur in normal NK cells or nonhemopoietic cells (43). Hence, the complexity of receptor signaling relies not only on the possibility that individual receptors interact one with each other, but also on a differential array of distinct subunits that may represent a hallmark of that specific cell type.

Our current study also explains what we previously reported on an atypical X-SCID phenotype and severe short stature associated with GH hyporesponsiveness and abnormal GHR-induced protein tyrosine phosphorylation (14), and indicates that growth failure in X-SCID is directly related to the genetic alteration.

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Disclosures

The authors have no financial conflict of interest.

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Shared Signaling Pathways Between Endocrine and Immune System Receptors: The Model of Gamma Chain

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Abstract: The rapid expansion in the past two decades in the understanding of the molecular basis of a large variety of novel congenital immunodeficiencies has provided valuable information on the signal transduction general mechanisms, that goes far beyond the comprehension of the individual disease. In most cases, the altered molecules are exclusively expressed in haematopoietic cells, while in other cases they are not restricted to a certain cell type. This leads to more complex clinical phenotypes, which contribute to unravel previously unappreciated non-haematopoietic functions of signaling proteins and the mechanism of coordination and integration of several pathways. Moreover, this knowledge will help define potential new therapeutic strategies through novel molecular targets, drive stem cell development into the desired differentiation path and ameliorate our comprehension of tissue engineering. This review focuses on the multiple roles in haematopoietic and non-haematopoietic receptors of the gamma signaling element with a special attention paid to the participation of gamma to growth hormone receptor signaling, confirming the presence of an interplay between endocrine and immune system.

Key Words: Congenital immunodeficiencies, growth hormone receptor, signal transduction, gamma chain, STATs.

1. INTRODUCTION

Endocrine and immune systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effective functions in an appropriate fashion. Cytokines and growth factors transmit signals through cell-surface receptors to the nucleus, activating intracytoplasmatic signaling molecules, ultimately resulting in the activation of specific transcription factors.

In the recent years, the description of complex phenotypes, in which immunodeficiency and growth failure were associated at a different extent greatly contributed to define that several signaling molecules play a role in both Growth Hormone (GH)-related and cytokines' signaling pathways. In fact, mutations of gamma chain (γc), Signal Transducers and Activators of Transcription 5 b (STAT5b), Nuclear Factor-κB (NF-κB) gene have been observed in patients with short stature due to GH insensitivity (GHI) and immunodeficiencies [1-7].

Recently, Adriani *et al.* demonstrated that the common cytokine receptor γc is required for a proper GH mediated STAT5b activation in B cell lines (BCLs) [8]. This study suggests a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, consistent with the presence of γc in non-hematopoietic tissues and underlines that immune and endocrine systems share signaling molecules.

This review, taking advantage of these complex human disorders, will focus on the relationship between different receptors that share common transducing elements and on potential diagnostic and therapeutical implications of such interactions.

2. THE GAMMA CYTOKINE TRANSDUCING ELEMENT, A SHARED COMPONENT OF SEVERAL CYTOKINE RECEPTORS

A wide number of cytokines have been molecularly recognized. They are soluble elements that control the immune and the haematopoietic system [9]. Their pleiotropic and redundant functions are due to the various receptors expressed on multiple target cells. Cytokines' specific roles are, in fact, closely dependent on their recognized targets. A contribution to a better understanding of such cytokine functions came out from the molecular characterization of a number of cytokine receptors. These receptors are classified into five families, created on the basis of extra- and intra-cellular domains structure affinity: the cytokine receptor superfamily, Interferon (IFN) receptor family, Tumor Necrosis Factor (TNF) receptor family, Tumor Growth Factor- (TGF)-β receptor family, and Interleukin (IL)- 8 receptor family. The cytokine receptor superfamily is the largest one, containing not less than 18 different receptor molecules. The common cytokine receptor γc is a shared component of several of these receptors, such as those for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [9]. Essentially, the γc and gp130 molecules contribute to increase the ligand-binding affinity and to establish an efficient intracellular signal transduction (Fig. 1).

Most of the information so far available on the role of γc came out from studies on X-Linked Severe Combined Immunodeficiency (SCID-X1) in humans and in mice carrying mutations in the γ gene [10,9,11].

The signals, that are dependent on γc, have been shown to mediate various immunological functions. The characterization of cytokine-activated genes, including genes regulated by γc-dependent cytokines, has been for a long time an area of considerable interest, although only few systematic studies have been conducted [12].

Even if the role of different cytokine-dependent signaling pathways in the regulation of gene responses is still unresolved, there is substantial evidence that γc controls the immune response at different, but often overlapping, checkpoints. IL-2, IL-7, and IL-15 were reported to regulate a highly overlapping set of more than one hundred overlapping genes, exerting different effects correlated to T-cell survival, activation and clonal expansion, and to the development and preservation of cell memory [13]. Moreover, IL-7 regulates lymphocyte development and homeostasis and it has effects on both T- and B-cell biology [14-16]. Although IL-7 receptor (IL-7R) is highly expressed on resting T cells, it is rapidly down-regulated by T-cell receptor (TCR) stimulation as well as by IL-2, thus supporting the idea that other cytokines are more relevant for effector functions [17]. In particular, IL-2 and IL-15 exert an important effect on promoting T-cell growth *in vitro*. In the effector phase of immune response, they can stimulate the proliferation of Natural Killer (NK) cells as well as induce the NK cell cytolytic activity. However, an important difference in the actions of these two cytokines has emerged *in vivo*, in particular, with regard to NK-cell development and $CD8^+$ T-cell homeostasis [18]. In fact, mice deficient in IL-2Ra or IL-2Rb have almost normal number of T, B and NK cells, thus indicating that IL-2 does not play a necessary role in cell development. Indeed, mice deficient in either IL-15 or IL-15Ra lack NK cells, confirming the role of IL-15 in NK-cell development [19,20]. Similarly, IL-15 is essential for the homeostatic prolifera-

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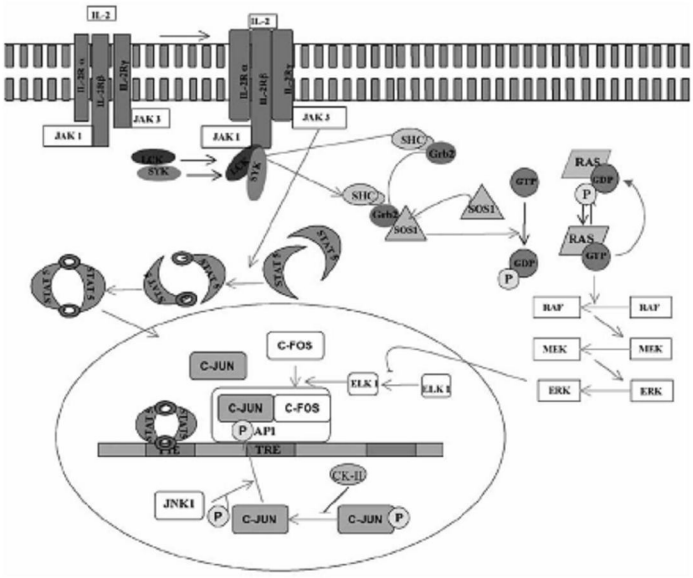

Fig. (1). Signaling pathway of IL-2 receptor.

tion of memory $CD8^+$ T cells and the maintenance of the steady-state level of $CD8^+$ T-cell memory.

IL-9 is a mast-cell growth factor. In IL-9-deficient mice, the lymphoid compartment develops normally. However, these mice exhibit excessive mucus production and mast cell proliferation [21]. But, such abnormalities have not been reported in humans with SCID-X1, suggesting that the γc is not responsible *per se* of this phenomenon.

Gene knockout (KO) models of γc-containing receptors for IL-4 and IL-21 have revealed a role in the regulation of immunoglobulin (Ig) production. Previous studies indicated a role for IL-4 in B-cell Ig class-switch to IgG1 and IgE [22].

It is now clear that cytokines acting through γc-containing receptors regulate several aspects of immune activation and play an important role in supporting either survival and proliferation or effector functions of already activated immune cells. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a balanced act of all γc-dependent cytokines seems to play a crucial role, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system.

The most important function shared between all γc-containing receptors seems to be the mitogenic effect. It is presumable that, given the widespread use of γc in different tissues, this pathway exerts its mitogenic and anti-apoptotic functions in a variety of distinct contexts (Table **1**) [23].

3. PARTICIPATION OF γC TO GH-R SIGNALING

Recent evidence indicates that γc participates to growth hormone receptor (GH-R) signaling, as well [8]. A previous observation was reported on a patient affected with SCID-X1 due to mutation of γc, who also had peripheral GH hypo-responsiveness associated with abnormalities in the protein phosphorylation events that

Table 1. Main Immunological Features of γc-Dependent Cytokines/Receptors

	Knockout Mice	Immunological Features
IL-2	IL-2, IL-2Rα	Normal T and B and NK cell development; defects in the function of T-regulatory cells
IL-4	Il-4	Normal T and B cell development; impaired Th-2 type responses
IL-7	IL-7, IL-7Rα	Reduction in the number of thymocytes, bone marrow B cell precursors, and peripheral B, CD4 and CD8 T cells
IL-9	IL-9	Normal development of lymphoid compartment. Increased mast cell proliferation
IL-15	IL-15, IL 15Rα	Reduction of number of NK cells, intestinal intraepithelial lymphocytes (IELs), memory CD8 T cells.
IL-21	IL-21Rα	Normal number of NK, T and B cells. Decreased levels of IgG1

normally occur following GH-R stimulation [7]. This observation, along with the abundance of γc in non-haematopoietic tissues, led to hypothesize that the GH-R signaling also involved γc. Taking advantage of this observation, our group extensively studied the potential role of γc in GH-R signaling using BCLs from healthy control and γc-negative SCID-X1 patients. In particular, we demonstrated that various GH-induced events were impaired in γc-negative BCLs. First, while GH enhanced proliferation of control BCLs in a dose-dependent fashion, the functional response to GH of the BCLs of γc-negative patients was severely impaired despite a comparable cellular expression of GH-R molecules. Furthermore, the signal transduction properties of GH-R following GH stimulation in SCID patients was abnormal as compared to control BCLs and, in particular, in the pattern of protein tyrosine phosphorylation. In fact, in contrast to what observed in control BCLs, in patients cells GH stimulation failed to induce phosphorylation of STAT5 molecule [8]. This blockage in GH-R signaling was specific of STAT5 in that other molecules, as Janus kinase (JAK) 2, STAT1, extracellular signal-regulated kinase (ERKs) and STAT3 were normally phosphorylated following GH stimulation of B cells, thus suggesting the presence of pathways independently regulated. In support of this, transduction of patients' cells with the wild type γc gene corrected the functional and biochemical abnormalities, resulting in an appropriate nuclear translocation of STAT5 after GH stimulation. These data suggest that the γc is an important subunit of the GH-R signaling complex in BCLs, where it is required for STAT5 phosphorylation and nuclear translocation, and not for the activation of other molecules of GH-R signaling apparatus (Fig. **2**).

It is to note, that the immunological reconstitution of SCID-X1 patient through bone marrow transplantation paralleled the restoring of GH-R functionality, which resulted in a normal production of Insuline Growth Factor I (IGF-I) [24]. 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.

Whether the involvement of γc to the GH-R apparatus confers some additional properties to this receptor in haematopoietic cells and the specific functions in immune cells remain to be elucidated. In $CD34^+$ progenitors, γc participates in haematopoietic cell differentiation by interacting with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) receptor-β (GM-CSFRβ)[25]. This interaction does not occur in normal NK cells or non-haematopoietic cells. Hence, the complexity of receptor signaling relies not only on the possibility that individual receptors interact one with each other, but also on a differential array of distinct subunits that may represent a hallmark of that receptor apparatus in a specific cell type.

4. GROWTH HORMONE RECEPTOR SIGNALING

4.1. Signal Transduction Through GH Receptor

Endocrine and immune systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effective functions in an appropriate fashion.

Cytokines and growth factors transmit signals through specific cell-surface receptors to the nucleus by activating intracytoplasmic signaling molecules. This process ultimately results in the activation of transcription factors.

GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function. More than 400 functions

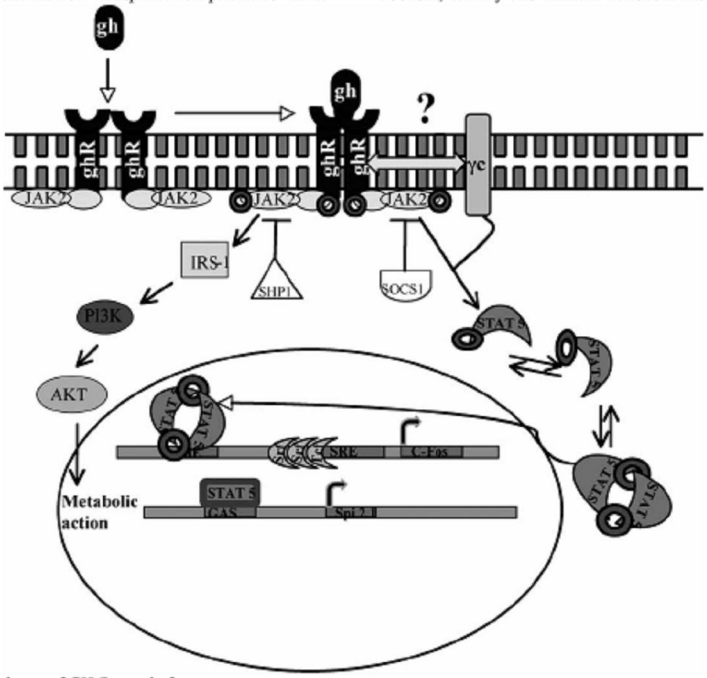

Fig. (2). Signaling pathway of GH-R: a role for γc.

linked to GH are long recognized. These 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 [26]. The GH-R was the first member of the cytokine receptor superfamily to be cloned [27]. It consists of a transmembrane protein and an extracellular domain that contains six cysteines linked by disulfide bonds and one free cysteine [28]. Similarly to other members of the cytokine/hematopoietin receptor superfamily, the intracellular region contains two motifs. A proline rich motif, referred to as box 1, is situated in the proximity of the membrane and consists of eight amino acids. The other cytoplasmic motif (referred to as box 2) mainly consists of hydrophobic amino acids along with acidic amino acids. 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 box 1 in the proximity of the GH-R cytoplasmic domain [29]. JAK2 belongs to the Janus family of cytoplasmic tyrosine kinases that includes JAKl, JAK2, JAK3, and tyrosine kinase 2 (TYK2). JAK2 activation is initiated by GH-induced receptor dimerization/oligomerization, which induces conformational changes resulting in JAK2 transphosphorylation and activation. The central role of JAK2 in this pathway is supported by the studies in which mutated GH-Rs fail to bind or activate JAK2, as clearly shown in several cell lines expressing truncated or mutated receptors [29,30]. These mutants fail to elicit GH dependent tyrosine phosphorylation of Src homologous and collagene-like (SHC) proteins, mitogen-activated protein kinases (MAPK), also designated as extracellular signal regulated kinases. After phosphorylation of JAK2, the receptor itself and several intracytoplasmic molecules are promptly phosphorylated on key tyrosine residues. Further signaling proteins recruited to JAK2/GH-R complex and/or activated in response to GH include: (1) SHC proteins that presumably lead to the activation of Ras/MAP (Rat sarcoma viral oncogene homolog/ Mitogen Activated Protein) kinase pathway; (2) insulin receptor substrates that have been implicated in the activation of phosphatidylinositol-3-kinase and the kinase AKT/PKB (Akt-8 retroviral oncogene/Protein kinase B); (3) phospholipases that lead to formation of diacylglycerol and activation of protein kinase C (PKC); and (4) 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. GH is able to activate NF-κB pathways, leading to the expression of cell cycle mediators [31]. This process ultimately results in the activation of multiple transcription factors as STAT1, STAT3, STAT5a, and STAT5b, as detailed in the next paragraph. The tyrosine-phosphorylated STAT 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 [32].

The duration of GH-activated signals is a key factor in relation to the biological actions of the hormone. Removal of cell surface GH-Rs by endocytosis is an early step in the termination of GH-dependent signaling. Furthermore, suppressors of cytokine signaling proteins (SOCS) act as negative regulators of the main cytokine-activated signaling pathway, the JAK/STAT signal cascade. However, since the activation of GH-dependent signaling pathways is mainly based on protein phosphorylation on tyrosine, serine, or threonine residues, the obvious mechanism for deactivation of this process is the recruitment of a protein tyrosine phosphatase to GH-R/JAK2 signaling complex [33]. This phosphatase would dephosphorylate GH-R, JAK2, or the STATs themselves leading to signaling down-regulation. Several studies documented that there are at least three different phosphatases involved in the specific down-regulation of GH-R signaling: (1) SH2 domain-containing protein-tyrosine phosphatase 1 (SHP1 also known as PTP-1); (2) PTP1b; and (3) PTP-H1 [34]. Taking into consideration that a so huge number of GH actions are direct, a deep understanding of the regulatory mechanisms that control in an integrated fashion, presumably, distinct signaling pathways related to the same receptor through activation/down-regulation processes is mandatory. This knowledge would help understand tissue specificity of GH action and would allow to 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 or inhibit the beneficial effects of GH [33].

4.2. STAT Family

STATs factors represent a family of cytoplasmic proteins that participate in gene control in response to cell stimulation with various extracellular polypeptides [34]. So far, seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 have been characterized [34]. These transcription factors mediate several functions by regulating the expression of effector genes involved in cell differentiation, survival, and apoptosis. All the STAT family members share in their structural arrangement functional motifs, and, in particular, the amino terminus that has a role in STAT dimerization. In addition, other motifs are a coiled-coil domain, involved in interactions with other proteins, a central DNA-binding domain, a SRC homology 2 (SH2) domain, a conserved single tyrosine residue that is phosphorylated following activation, and a carboxyl terminus that facilitates transcriptional activation.

In the cell biology, STAT proteins are essential regulators of cell proliferation, differentiation and survival in different cellular contexts, thus revealing their critical role in malignant transformation. Dysregulated STAT activation leads to increased angiogenesis and enhanced survival of malignant cells [35]. 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.

Recent studies have shown that JAK–STAT signaling can be regulated through distinct mechanisms. Studies on STATs 1, 3 and 5 have elegantly demonstrated that unphosphorylated STATs shuttle between the cytoplasm and the nucleus in the absence of cytokine activation. Differently, the phosphorylated STATs are retained in the nucleus and are only released upon dephosphorylation through nuclear phosphatases [32]. STATs are activated by a number of cytokines, including interferons and interleukins, as well as growth factors and hormones. STAT1 is inducible by IFN -α/β and IFN-c, and is involved in anti-viral and anti-bacterial response, in growth inhibition, apoptosis, and tumor suppression [34]. STAT1 mediates the anti-viral and immune/inflammatory effects of IFNs through the induction of immune effector and inflammatory genes, such as major histocompatibility complex (MHC), costimulatory molecules, chemokines, complement, inducible nitric oxide synthase, and Fcγ receptor I (FcγRI) genes. STAT1 is also important in host anti-tumor responses and is also involved in non-immune functions, such as the regulation of bone formation and destruction. STAT3 is mainly activated by cytokines and growth factors, including IL-6 family members and epidermal growth factor (EGF), and is involved in mitogenesis, survival, anti-apoptosis, and oncogenesis. STAT3 is also required for optimal IL-2-induced T-cell proliferative responses by up-regulating the expression of IL-2Rα, a component of the high-affinity IL-2 receptor [36]. STAT3 is required for early development, as STAT3 null alterations in mice were embryonically lethal. Subsequent tissue-specific gene targeting showed STAT3 functions in wound healing in keratinocytes, regeneration of liver, mammary involution, and survival of many cell types [36]. Recently, tissue-specific deletion of STAT3 in

bronchiolar and alveolar epithelial cells resulted in enhanced apoptosis in these cells following adenoviral infection, suggesting a cytoprotective function of STATs. STAT4 is predominantly stimulated by IL-12 and is involved in Th1 (T helper 1) development in humans. STAT4 is activated by IL-23 in murine cells as well, and, additionally, by IFN-α in human cells, being recruited to type I IFN receptor through interaction with STAT2 [34]. As it may be expected, STAT4-deficient mice exhibit an impaired Th1 differentiation, IFN-γ production, and cell-mediated immune responses. STAT6 molecule is activated by IL-4 and participates in Th2 (T helper 2) development [35]. STAT5A and STAT5B are involved in prolactin and growth hormone signaling, respectively.

Multiple inhibitory mechanisms down-regulate cytokine-JAK–STAT signaling. Down-regulation of this pathway is likely to be important for homeostasis and the prevention of chronic inflammation or autoimmunity. Both constitutive inhibitory pathways and inducible mechanisms have been described. These mechanisms can act at several levels in cytokine signaling, targeting or the receptors, or JAK proteins, or the STATs themselves. Constitutive inhibitory mechanisms include proteolysis, dephosphorylation, and interaction with inhibitory molecules termed Protein Inhibitors of Activated STATs (PIAS). Three major regulated or inducible inhibitory mechanisms have been identified, mediated by down-regulation of receptor expression, through the induction of inhibitory molecules termed SOCS proteins, and by rapid MAPK or PKC-dependent modification of pre-existing signaling components. One proposed mechanism for degrading cytokine signaling components, in which STATs are mostly involved, is the coupling of signaling proteins to proteasomes by SOCS molecules. SOCS family of proteins consists of eight members. These SOCS proteins are generally expressed at low levels in unstimulated cells and become rapidly induced by cytokines, thereby inhibiting JAK–STAT signaling and forming a classic negative-feedback loop [33]. Finally, the modulation of JAKs and STATs by various protein modifications and the cross-talk between different JAK–STAT pathways and other cellular signaling pathways provide additional levels of regulation of cellular homeostasis [33].

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.

Once GH-R signaling is activated, 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.

The effects of GH on growth are mostly mediated by intermediate factors, of which the IGF-I is the most studied [37]. GH upon binding to its cognate 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). The binding protein and the acid labile subunit (ALS) prolong the half-life of IGF-I and regulates its tissue distribution and bioavailability at the cellular level [38]. The linear growth is a complex process. GH and IGF-I participate as combined factors or separately to this process [39]. The double KO IGF-I + GH-R mouse revealed that 35% of body growth was due to IGF-I alone, 14% to GH alone and 34% to a combined effect of GH and IGF-I [40]. Despite the close relationship between GH and IGF-I, GH appears to have many cellular effects that are independent of IGF-I, and are most probably direct effects of GH mediated by its cognate receptor [39]. Moreover, all tissues but the liver express IGF-I transcripts in the absence of a functional GH-R, thus indicating that the molecule has additional biologic effects that are GH independent [39].

In addition, evidence supports a role for GH acting as a cytokine in the immune system under conditions of stress, counteracting immunosuppression by glucocorticoids [31]. 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 by the production of IGF-I, promote cell cycle progression and prevent apoptosis of lymphoid cells as well as a wide variety of other cells.

A so huge number of distinct biologic functions of the GH/IGF-I axis may be expressed through the existence of many separately regulated signaling pathways, as above described. Baixeras *et al.* have recently demonstrated that both GH and IGF-I are able to promote cell survival and proliferation through independent and different pathways, thus indicating a potential function related specificity of an individual pathway [41]. The effects on the proliferation were shown to be mainly mediated by the PI-3 kinase /Akt pathway and the transcription factor NF-kB. Moreover, GH regulates the expression of several cell cycle mediators, such as Bcl-2, cMyc and cyclin proteins [31]. The transcription factor NF-kB is involved in the regulation of many functions of the immune system, and its activation by GH would be one of the keys explaining the cytokine-like effects of GH on the immune system. Since NF-kB is related to the transduction of survival signals by GH, Jeay *et al.* 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 [31].

In addition to GH, also IGF-I, in turn, activates specific cellular pathways, through tyrosine kinase activation and phosphorylation, leading to the various biological actions. IGF-I exerts many 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 poorly characterized mechanisms [42]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factors [38]. The antiapoptotic function of IGF-I is mediated by IGF-I receptor (IGF-I-R). Many studies have clearly illustrated that IGFBPs can affect cell cycle progression and apoptosis by preventing IGF-I-R activation [38]. Thus, IGFBP-3 can also modulate the antiapoptotic effect of IGF-I by regulating the IGF-I/IGF-I-R interaction. Modulating IGFBPs by proteolysis also plays a role in regulating their proliferative effect. There is also evidence that IGFBP-3 can induce apoptosis on its own [38,43].

The liver is thought to be the major source of IGF-I. Liver deficient IGF-I (LID) mice show a major (75%) reduction in circulating IGF-I levels, whereas the expression in non-hepatic tissues was normal [44]. However, these mice are similar to wild-type mice with respect to body weight, body length or femoral length, thus indicating that the normal growth is mediated by autocrine/paracrine actions of IGF-I combined with non-hepatic sources of circulating IGF-I [44]. In humans, we documented that stem cell transplantation, performed to treat a severe combined immunodeficiency associated with a remarkable peripheral hyporesponsiveness to GH, was capable to restore IGF-I levels to normal values, thus adding in favour of a lymphocyte source of the mediator [24].

5. COMPLEX CLINICAL PHENOTYPES AFFECTING BOTH IMMUNE AND ENDOCRINE SYSTEMS

5.1. Murine Models

The Murine Model of SCID-X1

KO mice for the γc of the IL-2R represent the human counterpart of the SCID-X1 phenotype. These animal models show significant reduction of T, B, and NK cells associated with normal thymic architecture but reduced thymic cellularity. Thymic cellularity is

controlled by γc-dependent cytokines at the level of pro- and pre-T-cell development. In particular, each stage during pro- and pre-T-cell development involves γc cytokines for a proper T-lymphocyte production. Among T-cell developmental stages, the transition from stage 2 ($CD44^+CD25^+$) to stage 3 ($CD44^-CD25^+$) pro-T-cell, seems to be the critical stage for γc. In fact, in γc-deficient mice a higher number of pro-T cells is observed. Moreover, the few thymocytes in γc-deficient mice exhibit a relatively normal pattern of CD4 and CD8 expression. However, an elevated number of $CD4^+$ T cells is seen in periphery differently from $CD8^+$ T cells that are expressed at low level. This observation suggests that γc could be implicated in the regulation of thymic export and/or in the homeostasis of the peripheral T, as well.

Even if human and mice models of SCID-X1 are similar in the decrease of T and NK cells, there is marked difference in B-cell development. In fact, differently from what observed in the animal model, human SCID-X1 shows normal or high level of B cells, as also observed in IL-7 deficient mice. However, in spite of such differences, SCID-X1 mice represent an important model to better understand the pathophysiology of the human disease.

The Murine Model of STAT 5 Knock Out

Most of the current knowledge about the biological function of STAT family members has been achieved through disruption approaches and studies of KO mice [45]. In particular, all seven mouse STAT genes have been disrupted in transgenic animals [46]. Most of these animals have been studied and the results obtained have reinforced the notion that STAT proteins are a critical point of cytokine signaling specificity [47]. Unlike most of the STATs, the two STAT5 proteins are activated in reply to a variety of cytokines as well as tyrosine kinase receptors. Then, it was supposed that STAT5a/b would have very fundamental functions in regulating cell growth.

Differently from the other STATs, the study of the STAT5 function *in vivo* has been more difficult because there are 2 isoforms, STAT5a and STAT5b, very close on the chromosome 11 [7]. This position made quite difficult to create a double KO. In particular, these two proteins are 96% related at the amino acid level, but diverge, principally, at the carboxyl terminus. However, the diversities led to different DNA-binding specificities related to a single amino acid variation in the DNA-binding field. Nevertheless, these two proteins control the expression of common as well as distinct genes. Moreover, slight difference in the tissue allocation of their mRNA has been found, thus contributing to their functional specificity. At first, STAT5a and STAT5b KO were individually produced and examined.

STAT5a gene targeting in mice confirmed its principal role in mammopoietic and lactogenic signaling [46]. In fact, the phenotype of STAT5a deficient mice showed impaired mammary gland development and lactogenesis. STAT5a KO mice developed normally and were indistinguishable from hemizygous and wild-type littermates in size, weight and fertility [47]. Unlikely STAT5a KO, 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 growth hormone receptor deficient mice, predominantly resulting in failure of postnatal growth development. STAT5b mediates the sexual dimorphic effects of GH pulses in liver and other target tissues. This comes out from the observation that in STAT5b KO males body growth rates and male-specific liver gene expression were decreased to the levels observed in wild-type females. On the contrary, female-predominant liver gene products were increased to a level intermediate to those of wild-type male and female levels. A recent study, analyzing mice which lacked STAT5a and/or STAT5b gene, confirmed these findings [48]. In addition, STAT5a/b double KO mice were found to have a defect in the development of functional corporal lutea in the ovary, resulting in female infertility. Female infertility is not observed in the STAT5a or STAT5b KO mice alone, demonstrating the functional redundancy of the STAT5 proteins [46].

The role of STAT5 in the immune system was also 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 [49]. This is because STAT5a activation is responsible for IL-2-induced IL-2Rα expression in T lymphocytes. Although IL-2Rα expression is also defective in STAT5b KO mice, it cannot be corrected by administration of high doses of IL-2. Splenocytes from STAT5b KO mice exhibited greatly diminished proliferation in response to IL-2 and IL-15. Basal as well as IL-2- and IL-15-mediated augmentation of NK cytolytic activity was also greatly diminished in STAT5b KO mice. The percentage of NK cells expressing IL-2Rβ as well as the levels of IL-2- and IL-15-induced perforin expression in splenocytes were also significantly diminished in STAT5b KO mice. These data indicate that STAT5b is essential for potent NK cell-mediated proliferation and cytolytic activity. In addition, bone marrow-derived macrophages from STAT5a KO mice have a defect in GM-CSF-induced proliferation as well as in the expression of GM-CSF-dependent genes such as CIS and a bcl-2 like gene A1 [50]. One of the surprising results from disruption studies of either STAT5a or STAT5b was the observation that in either cases the development of the haematopoietic system took place apparently normally, in spite of the many studies showing STAT5 activation in response to several haematopoietic cytokines as erythropoietin, IL-2, IL-3, IL-5, GM-CSF, IL-7, and CSF-1. Of note, a few of these cytokines are implicated in haematopoietic development and differentiation. However, an impaired proliferation of peripheral T lymphocytes was observed in STAT5 a/b KO mice, even if this characteristic is due, probably, to a defect in the cell cycle entry rather than to a decreasing of IL-2 receptor expression. In fact, while lymphopoiesis was normal, T cells from double KO mice showed a marked failure to undergo cell cycle progression and a diminished expression of proteins fundamental for proliferation [47].

Thus, it is clear how STAT5 proteins are strongly correlated with some oncogenic events, such as proliferation and apoptosis [47]; so, the therapeutic inhibition of these transcription factors may be proven helpful for those diseases characterized by an alteration of cellular homeostasis.

5.2. Human Models

The Human Model of SCID-X1

Primary immunodeficiency syndromes represent a group of distinct genetic disorders inherited in an autosomal recessive or X-linked mode. It has become evident that most of the immunodeficiencies are disorders of the intra- or inter-cellular communication network. The most common form of SCID accounting for approximately half of all cases, with an estimated incidence of 1:150000 to 1:200000 live births, is the SCID-X1 [51,52]. This form of SCID is characterized by the absence of mature T and NK lymphocytes and absent immunoglobulin synthesis, despite the presence of a normal or sometime elevated number of B cells. In SCID-X1 patients, T cells are absent not only in the peripheral blood but also in the central and peripheral lymphoid organs suggesting an early block in the T-cell differentiation pathway [53]. Furthermore, although peripheral B cells exhibit a normal phenotype, SCID-X1 patients B cells are not functional, even after T-cell reconstitution by means of bone marrow transplantation [54,55]. Mutations of the γc gene are responsible for the SCID-X1 [56]. As above mentioned, the SCID-X1 is also associated with GH hyporesponsiveness that results in the impairment of linear growth [7].

The Human Model of STAT5 Mutations

STAT5b is involved in GH mediated IGF-I gene transcription and production of IGF-I and in transcription and production of IGFBP-3 and the Acide Labile Subunit (ALS) as well [57]. This came out from the observation of patients carrying mutations of STAT5b gene. To date, a total of six patients carrying genetic abnormalities of STAT5b have been identified [2,4,5]. Interestingly, five of the six cases are female, indicating that in humans, unlike mice, STAT5b is crucial in the growth of both males and females. Serum concentrations of IGF-I, IGFBP-3 and ALS were strikingly reduced, while basal and stimulated GH concentrations were either normal or increased. The clinical phenotype of these patients was characterized by growth failure and immunodeficiency. Recurrent pulmonary infections, chronic diarrhoea, severe eczema, herpes keratitis, severe varicella, juvenile arthritis, lymphoid interstitial pneumonia with fibrosis, were reported in these patients [1-5]. Several immune deficiencies, such as decreased number and function of $CD4^{+}CD25^{+}$ regulatory T cells, reduced up-regulation of CD25 and of the common γc cytokine receptor and perforin in response to IL-2 were documented, thus suggesting that STAT5b propagates an important IL-2-mediated signal for the *in vivo* accumulation of functional regulatory T cells [42]. However, the relationship between endocrine and immune dysfunctions in patients with STAT5b alterations are not yet completely defined, in that one male patient with a homozygous mutation of STAT5b with severe short stature did not suffer from any infection and did not show any immunological abnormality [58]. Of note, this patient was the only male described with STAT5b deficiency, thus suggesting the possibility that the phenotype is sex-dependent.

On the contrary, no immunodeficiency was observed in patients with low IGF-I due to GH-R mutation, IGF-I gene alterations [59,60], or mutations in the IGF-I-R gene [61]. This finding would mean that a number of distinct pathways under separate regulatory mechanisms cooperate in an integrated fashion to achieve a specific function.

As a matter of fact, gene disruption of additional signaling molecules implicated in GH-R signaling does not lead to a combined endocrinological and immunological disorder, thus implying that the integration of distinct pathways are responsible for a full expression of a certain function. For istance, targeted disruption of ERK1 in rodent models results in mice that are of normal size but defective in thymocyte maturation. In keeping with this, in humans normal GH-induced ERK signaling seems to be insufficient to compensate the reduced IGF-I expression due to absence of STAT5b or GH-R mutations [2,62]. Certainly, STAT5b seems a shared component between signaling pathways implicated in both immunological and endocrine functions. Many cytokines, as IL-2, IL-7, IL-21 and IFN-γ, can activate STAT5b. Hwa *et al*. have recently demonstrated the cytokine IFN-γ, like GH, could not activate mutant STAT5b, resulting in a markedly reduced expression of the IGF-I gene [3]. It is, therefore, likely that cytokines, important for cellular immunity such as IL-2 and IFN-γ, require STAT5b for efficient regulation of multiple genes, also including IGF-I [3].

The Human Model of NF-κB Mutation

As a further example of complex disease involving both endocrine and immune system, it has been recently documented that a heterozygous mutation of the Inhibitor of NF-κB (I-κBα) gene was implicated in a clinical phenotype characterized by severe immunodeficiency and clinical signs of partial GHI [6]. The NF-κB signaling pathway plays a crucial role in many physiological process, such as the innate and adaptative immune responses, apoptosis and inflammation [63]. NF-κB is bound to I-κB and his activation involves the phosphorylation of the inhibitor, which results in I-κB degradation and releasing of NF-κB. Free NF-κB translocates into the nucleus and activates transcription of target genes.

6. GH/IGF-I AXIS AND RISK OF CANCER

As for the relationship between the GH/IGF-I axis and the risk of developing cancer, no conclusive data are available. The risk of developing cancer is determined by a combination of genetic factors and environmental effects, in particular diet and lifestyle. There is increasing evidence that the GH/IGF-I axis may provide a link between these factors and the development of cancers through the regulation of normal cell proliferation, differentiation and apoptosis [64].

Recently, several epidemiological reports have been systematically reviewed to define the association between circulating IGF-I and IGFBP-3 concentrations and the risk of developing cancer [65]. Elevated serum levels of IGF-I have been associated with an increased risk of breast, prostate and colorectal cancer in humans in several epidemiological studies [66,67]. In the transformed cell, there are abundant data showing that IGF-I-R regulates cancer cell proliferation, survival and metastasis [68]. Targeted disruption of the IGF-I-R results in almost complete resistance of mouse embryonic fibroblasts to transformation, while inhibition of the IGF-I-R almost completely blocks colony formation by melanoma cells in soft agar and substantially reduces tumour formation in a mouse xenograft model [66]. IGF-I is mitogenic 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 [65]. However, the association between elevated concentrations of IGF-I and the increased risk of cancer is modest and varies between different sites.

On the opposite site, several studies have documented that other cancer risk factors, including diet and lifestyle factors, have an effect on IGF-I and IGFBP-3 concentrations [65].

Differently from IGF-I and IGFBP-3, the involvement of GH in the physiopathology of cancer is still questionable. As matter of fact, GH treatment positively influences in parallel both IGF-I and IGFBP-3, contributing to the maintenance of the balance between these two molecules. GH, through its receptor-linked signal transduction mechanism, stimulates the expression of several genes whose significance in the development of cancer is still unknown. Overall cancer incidence is not increased in acromegaly.

STAT molecules and, in particular, STAT3 and STAT5, have been demonstrated to directly participate in tumor development and progression [69,70]. 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. Moreover, tumor cells possessing activated STAT3 or STAT5 are predicted to be resistant to chemotherapeutic agents that may utilize similar apoptotic pathways pathway. It has been clearly documented that inhibition of constitutively active STATs result in growth inhibition and induction of apoptosis in tumor cells [70,71].

7. THERAPEUTIC PERSPECTIVES

As a matter of fact, the expression of more than 400 biologic effects functionally related to an individual receptor-ligand, GH/GH-R, may occur based on the assumption that an array of distinct pathways may be integrated in a tightly coordinated fashion. A primary aim of novel therapies should be to increase our understanding of the relationship between inhibitory and stimulatory actions of GH-related signaling pathways. 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. Thus, in principle it is conceivable to hypothesize a pharmacological intervention in tissue engineering and remodel-

ling by interfering in such pathways. The dissection of individual signaling pathways and the identification of the specific molecule implicated in that certain function remain the major goal of such novel therapeutic approaches.

The development of targeted therapeutics to activate growth inhibition (e.g. cancer) or stimulation (e.g. tissue engineering) represents the major 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. Drugs to disrupt IGF-I-R have been developed. Most of the anti-IGF-I-R strategies have been directed against the receptor itself. A different approach may be the removal of the ligand, or the use of tyrosine kinase inhibitors. This class of reagents are good candidate for the treatment of a number of different cancer types. However, because of the ubiquitous nature of IGF-I-R expression and action, blockade of IGF-I-R could affect multiple tissues. Therefore, careful attention to future clinical trials of these therapeutic targeting in combination with chemotherapy will be necessary [72].

The most potent therapy for reducing serum IGF-I levels is Pegvisomat, the GH-R antagonist. The potential role of this compound in the treatment of IGF-I-influenced cancers is intriguing. In animal models of metastatic colon cancer, Pegvisomat in combination with conventional chemotherapy virtually abolishes the metastatic disease [64]. It has also been reported to exert inhibitory effects against breast cancer cell lines implanted into athymic mice [73].

As above discussed, aberrant STAT activation is also associated with oncogenesis. Thus, the development of selective inhibitors of STAT activation may be a promising area in the field of novel anticancer therapeutics [70]. However, whether this can be translated into the clinical setting and used for the treatment of human cancers remain to be extensively proved. In the near future the first goal of studies in this field will be the identification of the individual molecule implicated in that specific GH related function. This may lead to targeted therapy aimed at potentiating or abolishing only that biologic effect.

The participation of γc to several receptors implicated in the immune response would also suggest potential effect of GH in immune response, even though the precise role of the hormone is not completely defined. It should be noted that overexpression of γc in engineered lymphocytes, used for gene correction of SCID-X1 and mutation of γc resulted in neoplastic transformation in 3 cases. Even though this side effect was interpreted as due to insertional oncogenesis, it is also conceivable to hypothesize that overexpression of the molecule exerts an oncogenic effect *per se*.

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ABBREVIATIONS

AKT	=	Akt-8 retroviral oncogene
ALS	=	Acide Labile Subunit
BCLs	=	B cell lines
EGF	=	Epidermal Growth Factor
ERK	=	Extracellular signal-regulated kinase
FcγRI	=	Fcγ receptor I
γc	=	Gamma chain
GH	=	Growth Hormone
GHI	=	Growth Hormone Insensitivity
GH-R	=	Growth Hormone Receptor
GM-CSF	=	Granulocyte Macrophage-Colony Stimulating Factor
GM-CSFRβ	=	Granulocyte Macrophage-Colony Stimulating Factor receptor-β
IFN	=	Interferon
Ig	=	Immunoglobulin
IGFBP	=	Insuline Growth Factor Binding Protein
IGF-I	=	Insuline Growth Factor I
IGF-I-R	=	IGF-I receptor
JAK	=	Janus Kinase
IL	=	Interleukin
LID	=	Liver deficient IGF-I
MAPK	=	Mitogen Activated Protein Kinase
MHC	=	Major Histocompatibility Complex
NF-κB	=	Nuclear Factor-κB
NK	=	Natural Killer
PIAS	=	Protein Inhibitors of Activated STATs
PI-3 kinase	=	Phosphatidylinositol-3-kinase
PKB	=	Protein Kinase B
PKC	=	Protein Kinase C
PTP	=	Protein Tyrosine Phosphatase
Ras	=	Rat sarcoma viral oncogene homolog
SCID-X1	=	X-linked Severe Combined Immunodeficiency
SH2	=	SRC homology 2
SHC	=	Src homologous and collagene-like
SHP	=	SH2 domain-containing protein-tyrosine phosphatase
SOCS	=	Suppressors of cytokine signaling
STAT	=	Signal Transducers and Activators of Transcription
TGF-β	=	Tumor Growth Factor-β
Th	=	T helper
TNF	=	Tumor Necrosis Factor
γc	=	γ chain
TCR	=	T Cell Receptor

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Conclusive remarks

The involvement of gc to the GHR apparatus confers some additional properties to this receptor in hemopoietic cells, but the specific functions remain to be elucidated. Of note, in CD34+ progenitors (HPC), gc participates in hematopoietic cell differentiation by interacting with GM-CSFRb. This interaction does not occur in normal NK cells or nonhemopoietic cells (31). Hence, the complexity of receptor signaling relies not only on the possibility that individual receptors interact one with each other, but also on a differential array of distinct subunits that may represent a hallmark of that specific cell type.

In conclusion, the evaluation of the existence of a previously unacknowledged link between individually well-studied elements, such as GHR and gc, confirm the cross-talk between receptor signaling systems as an emerging as well as important and exciting area of signaling research.

In lymphocytes, several functions have been attributed to GH. They include a mitogenic effect on T and B lymphocytes, help in cell development and lymphopoiesis of immune system (32) and potentiation of several functions, including NK activity. GH also plays an anti-apoptotic effect on peripheral lymphocytes (33-34). Thus, in principle it is conceivable to hypothesize a pharmacological intervention in tissue engineering and remodelling by interfering in such pathways. The dissection of individual signaling pathways and the identification of the specific molecule implicated in that certain function remain the major goal of such novel therapeutic approaches. The development of targeted therapeutics to activate growth inhibition (e.g. cancer) or stimulation (e.g. tissue engineering) represents the

major issue. Progress in defining the pathogenic implications of GHR downstream molecules in cell growth might lead to the development of novel targeting strategies to fight those cancers that may be proven responsive.

The participation of gc to several receptors implicated in the immune response would also suggest potential effects of GH in immune response, even though the precise role of the hormone is not completely defined.

§ 1.2 The common g chain provides spontaneous and GH-dependent cell cycle progression, related to its cellular amount

As already mentioned above, mutations of the *IL2RG* gene encoding the gc lead to the X-SCID (35-36). 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 immunological complications such as Graft-versus-host-disease (GVHD) associated to a potential long-term decline in immune cell functions. These difficulties encouraged gene therapy trials (37). This strategy using *ex-vivo* retroviral vectors has been proven as a corrective therapeutic approach for X-SCID in humans (38-43). Immunological reconstitution has been documented in 17 out of 20 patients enrolled in two distinct clinical studies (37, 41). Unfortunately, 5 of these patients developed a lymphoproliferative disorder (44-46), not observed in gene therapy trials for SCID due to ADA deficiency (47). This event was attributed to upregulated expression of the *LMO2* oncogene, as a consequence of insertional mutagenesis (48). However, it was clearly documented only in 2 cases. Even though the other patients may have the vector integration near *LMO2* or other oncogenes (48), it is also conceivable that the transgene could have a role *per se* in cell cycle

progression. In keeping with this hypothesis, overexpression of gc 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 (49). 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 gc control of cell cycle (50). These results suggest that insertional mutagenesis may not be the only cause of laeukemogenesis and that the expression level of *IL2RG* could influence the cell cycle progression directly or its effect being mediated by cytokines triggers.

It has been documented that gc interleukin receptors activity enhance leukemogenesis (51). In turn, this subunit activates several key signaling molecules, as JAK3, whose constitutive activation is frequently associated to autonomous cell growth and malignant transformation of lymphoid cells (52-53). Recently, as above mentioned, we demonstrated that gc subunit is also involved in GHR signaling in B lymphoblastoid cell lines (BCLs) (54).

In this second part of the study, we show trough gc 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.

In particular, to define an intrinsic mitogenic property of gc dependent on the amount of the protein, we used *in vitro* cellular models containing different amounts of gc. In particular, BCLs from normal subjects, cells transduced with lipid vector containing nontargeting short interfering RNA

(siRNA), BCLs transduced with siRNA to knockdown gc expression and BCLs from X-SCID patients were used. Since self sufficiency in growth has been suggested as one of the six acquired capabilities of cancer phenotype (55), we examined the abilities of these previously mentioned BCLs to grow in serum-deficient conditions and showed that gc silencing inhibits self-sufficient growth. Moreover, since JAK3 is essential for autonomous proliferation being physically linked to gc, we further investigated the role of gc in self-sufficient growth, evaluating JAK3 activation. We found that the reduction of the protein down-regulates constitutively activated JAK3 in BCLs.

It has been reported that GH enhances BCLs proliferation in vitro (56) and that gc is functionally linked to GHR (54). Moreover, it has been described the association between lymphoproliferative events and supra-physiological doses of GH, both in mice and humans (57). Since 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 (58), we evaluated the response to GH stimulation of X-SCID BCLs, control cells and gc-silenced BCLs to assess whether gc amount could influence GH response. We found that gc silencing also inhibits GH-induced cell proliferation. In this context, since the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (29, 59-62) and STAT5 is considered a transforming agent in lymphoma and other cell types (63), we then evaluated whether gc-silencing had effect on GH-induced STAT5 subcellular localization. The result showed that the reduction of gc amount also inhibits STAT5 activation and the subsequent its nuclear translocation, that follows GHR perturbation.

This study has been published on *The Journal of Immunology*, see the manuscript 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.

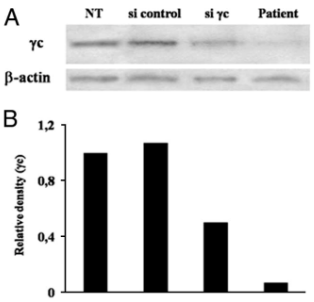

FIGURE 1. The γ_c silencing by siRNA induced a reduction in protein amount. *A*, After 96 h of culture, control BCLs transfected with nontargeting siRNA (si control), γ_c siRNA (si γ_c), or nontransfected (NT), and X-SCID BCLs (patient) were lysed and γ_c total amount was measured by Western blotting. 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*.

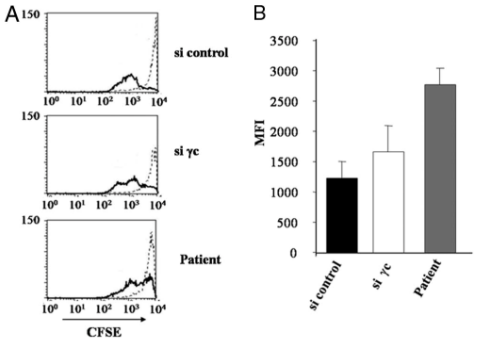

FIGURE 2. The γ_c was involved in self-sufficient growth of BCLs. *A*, After 12 h of starvation, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested 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*.

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

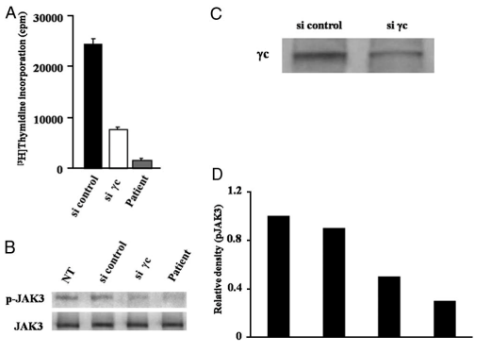

FIGURE 3. The γ_c protein depletion had effect on spontaneous cell proliferation and on activated JAK3 levels. *A*, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were tested for their ability to proliferate in serum-free medium. Cultures 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*.

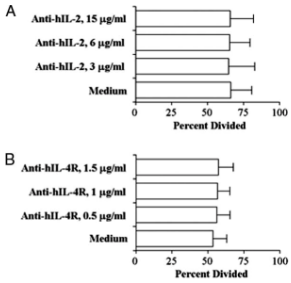

FIGURE 4. The γ_c-activating cytokines did not affect self-sufficient growth of BCLs. *A*, Control BCLs were treated with the indicated concentration of Anti-human IL-2, were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested and cell proliferation was assessed 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.

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

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

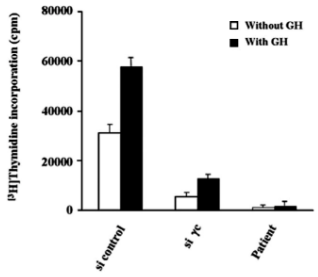

FIGURE 5. Silencing of γ_c inhibited GH-induced proliferation. Cell proliferation of BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) or nontransfected (NT) stimulated with recombinant GH (50 ng/ml) was evaluated through [^{3}H]thymidine incorporation assay. Error bar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

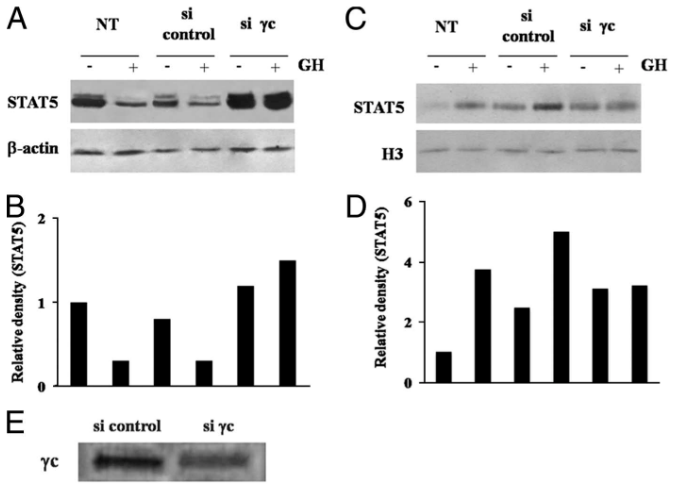

FIGURE 6. Silencing of γ_c influenced STAT5 nuclear translocation in B cell lines. Cells from BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were analyzed for subcellular localization of STAT5. Cells were stimulated with 500 ng/ml recombinant 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.

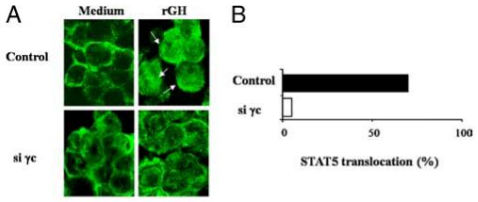

FIGURE 7. The γ_c silencing impairs recombinant GH-induced STAT5 subcellular redistribution. *A*, Evaluation of STAT5 subcellular localization through confocal microscopy. Control or γ_c-silenced BCLs (si γc) were cultured in the absence or presence of 500 ng/ml recombinant GH for 30 min. Arrows indicate 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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Conclusive remarks

These data add new evidence for a possible intrinsic mitogenic role of gc 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 gc 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 gc, a malignant lymphoproliferation occurred in 5 out of 20 patients enrolled into the trials, alarming the scientific community (37). To explain these adverse events, studies were conducted to define whether the retroviral insertional mutagenesis could have played a role. In 2 cases an aberrant transcription and expression of *LMO2* was clearly documented (48). 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 would imply that there is no evidence of continued abnormal growth of clones containing insertionally activated growth-promoting genes (64). 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 (65). Here, we demonstrate that gc 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 gc 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 (51). 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 (53). 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). 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 (68). JAK3 has also the capacity to activate DNA synthesis and protooncogenes, such as c-myc and c-fos (69).

In this study, we also observed that the participation of gc 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 gc could be favoured by the participation of gc in GHR signaling. 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 (57). Moreover, a putative role of GH as a cofactor in tumor growth is plausible, since several carcinomas express GHR (70). In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events (71).

Our data would imply that the expression levels of gc 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, gc is expressed at a normal extent in cells, transduced with retroviral vectors containing wild-type gc, 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 occurr in a third of the cases treated with lentiviral vectors containing wild-type gc (49). 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 (49). Furthermore, by searching in Mouse Retroviral Tagged Cancer Gene Database, integration in *Il2rg* has been found in 2 cases of retrovirally induced leukemia (72).

In conclusion, our data demonstrate a direct relationship between the amount of gc expression and its role in cell cycle progression. 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.

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CHAPTER 2

The Nude/SCID phenotype

In 1966, S.P. Flanagan identified a new mouse phenotype that spontaneously appeared in the Virus Laboratory of Ruchill Hospital, Glasgow, UK, characterized by loss of the hair. This mouse showed an abnormal keratinization in hair fibers, with follicular infundibulum unable to enter the epidermis (1). These affected mice also showed an inborn dysgenesis of the thymus resulting in a compromised immune system lacking T cells (1).

Subsequently, the molecular nature of the nude defect was characterized and attributed to a genetic alteration of the transcription factor Forkhead box (Fox) Foxn1 (also called Whn or Hfh11), mainly expressed in thymus and skin (2-4). In addition, spontaneous mutations in the *Foxn1* gene also occur in rats (4). The rat '*Whn*' locus is located on chromosome 10, and homozygous nude rats are phenotypically comparable to nude mice (5). The analysis of the genomic sequence of the nude mouse revealed the presence of a single base-pair deletion in exon 3, absent in the wildtype allele. This deletion led to a frameshift that resulted in an aberrant protein prematurely terminating in exon 6 and the loss of the postulated DNA-binding domain.

To verify that mutations of the identified gene were responsible for the nude phenotype Su et al. generated a Foxn1 allele, termed Foxn1-delta, that encoded a transcript lacking exon 3, resulting in a 154-amino acid deletion from the 285-residue N terminus of the protein (6). Mice homozygous for this allele showed abnormal thymic architecture, lacking cortical and medullary domains. In contrast to thymi from nude mice lacking Foxn1, thymi from mice homozygous for Foxn1-delta promoted T-cell development but with

specific defects at both the double-negative and double-positive stages. Su et al. concluded that initiation and progression of thymic epithelial cell (TEC) differentiation were genetically separable functions of FOXN1 and that the N-terminal domain is required for cross-talk-dependent TEC differentiation.

The mouse *nude* mutation led to an abnormal development of the skin and thymus (1, 7) and a severe alteration of the nails (8). Later studies demonstrated that both defects, as lack of fur development and agenesis of the thymus, are pleiotropic effects of the same gene (9-10). In particular, the skin of the nude mouse contains the same number of hair follicles as a wild type control, but these follicles result in an uncomplete hair, that could not enter skin surface (1, 8). Flanagan analyzed carefully nude mouse skin and observed that at birth the hair follicles were normal, but by 6 days after birth the hair started to twist and coil, failing to penetrate the epidermis (1). This hairless condition could be reverted by oral administration of cyclosporine A or recombinant keratinocyte growth factor (KGF or FGF-7), that influence the number of hair follicles or the cyclic hair growth (11). Furthermore, the nude mouse epidermis shows failure in differentiation and a reduced number of tonofilaments are observed in spinous, granular and basal layers (8). The nude *Foxn1* gene doesn't affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle (12-13).

In addition to these cutaneous abnormalities, nude animals develop an abnormal thymus, resulting in a severe T-cell deficiency and an overall severely impaired immune system. In fact, thymus morphogenesis is stopped at the first stages with no subcapsular, cortical, and medullary regions formation, which characterizes a normal mature organ (10). In addition, the

observation that thymus restoration doesn't lead to hair growth demonstrated that the lack of the hair and the athymia were not related one with each other (9-10).

Furthermore, nude phenotype is characterized by nail malformations and poor fertility. The first condition is, probably, due to an abnormal production of filaggrin protein in nail matrix and nail plate, subsequent to a loss of Keratin 1 protein. Instead, the second condition may be the result of changes in hormonal status, as demonstrated by altered serum levels of estradiol, progesterone and thyroxine (8).

In nude mice the thymic agenesis is congenital and it closely resembles thymic agenesis of newborns affected with DiGeorge syndrome (11). DiGeorge syndrome occurs spontaneously and children with this syndrome are characterized by thymic hypoplasia or aplasia. Unlike the association of hairlessness in the nude mouse, DiGeorge syndrome was found to be often associated to neonatal tetany and major anomalies of great vessels. These latter defects, due to malformation of the parathyroid and heart, derived from a major embryologic defect in the third and fourth pharyngeal pouch from which the thymus primordium emerged. Children with the DiGeorge syndrome also manifest lymphopenia, reduced number of T cells, poorly responsive to common mitogens (14). These similarities led to an erroneous interpretation of DiGeorge syndrome as the human counterpart of nude mouse phenotype for many years until the first description of the human equivalent of the Nude/SCID phenotype, which represents the real athymic human model.

§ 2.1 Novel immunodeficiencies: the model of the human Nude/SCID phenotype

More than 30 years from the original description of spontaneous murine disease, the first human equivalent of the Nude/SCID phenotype was described in 1996. The identification of the human counterpart of nude mouse phenotype began with identification of two sisters, whose clinical phenotype was characterized by congenital alopecia, eyebrows, eyelashes, nail dystrophy and a several T-cell immunodeficiencies, inherited as an autosomal-recessive disorder (15). The T-cell defect was characterized by severe functional impairment, as shown by the lack of proliferative response. The functional abnormality occurred in spite of the presence of a few phenotypically mature T cells, thus suggesting the qualitative nature of the defect (15). Since alopecia and nail dystrophy are also found in other syndromes, such as dyskeratosis congenita (DC) (16-17), other signs of this disease were investigated. Differently from DC, major signs, such as abnormal pigmentation of the skin and mucosal leucoplakia, were lacking in Nude/SCID and the immunological abnormalities were different from those reported in patients with DC in both severity of clinical course and type of alterations (18-19). Moreover, both patients showed alopecia at birth and in one sib it still persisted after bone marrow transplantation, thus ruling out that it was secondary to acquired skin damage. This finding suggested that the alopecia in this patient was primitive in nature (20). Furthermore, these features were similar to those reported in athymic mice, that completely lack body hair and in which restoration of a thymus did not lead to hair growth (21). Taken together, these observations suggested an association between

alopecia and the immunodeficiency reported in the 2 sisters, linked to a single gene defect (15).

In humans, *"FOXN1"* is located on chromosome 17 (22) and it encodes a transcription factor mainly expressed in the epithelial cells of the skin and thymus, maintaining the balance between growth and differentiation. Later, a molecular analysis in these patients was performed and revealed the presence of a C-to-T shift at 792 nucleotide position in the sequence of the *FOXN1* cDNA that leads to a nonsense mutation R255X in exon 5, with a complete absence of a functional protein (22) similar to the previously described rat and mouse *Foxn1* mutations (23-25).

The FOX gene family comprises a diverse group of 'winged helix' transcription factors that are involved in development, metabolism, cancer and aging. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions (26). They were first recognized in *Drosophila*, but later they were also identified in other organisms, from *yeasts* to *humans*. The term FOX is now used to refer to all chordate forkhead transcription factors. A phylogenetic analysis led to classify all known FOX proteins in at least 15 subfamilies (named from A to Q) (27) on the basis of their structure; in each subfamily (or class), an individual gene is identified by a number. The crystal structure of the forkhead DNA binding domain is a 'winged helix' motif, consisting of three a helices flanked by two 'wings' of b strands and loops (28). The structure and the amino acids sequence are highly conserved within species and family members. The functional effect of all FOX proteins can be either the activation (transactivation) or the inhibition of gene transcription33 in a wide range of context. *Fox* gene mutations can be associated with diverse

phenotypes as cranio-pharyngeal developmental defect (*FOXE1*), speech and language abnormalities (*Foxp2*) and hearing loss (*Foxj1*) (29). Moreover, most of these winged helix proteins play crucial roles in several aspects of immune regulation. In particular, genetic alterations of at least four FOX family members, FOXP3, FOXJ1, members of the FOXO subfamily and FOXN1, result in paradigmatic immune disorders and well defined novel clinical entities (30).

FOXP3 (scurfin, sf, JM2) is the most studied forkhead family member in immunology, because of its role in the pathogenesis of autoimmunity associated with immunological functional disorders (31-33). FOXP3 was found to be expressed in CD4+ CD25+ regulatory T-cells (Treg), that represent a subset of CD4+ T-cells bearing high levels of CD25 (the IL-2 receptor a-chain), whose role is to maintain self-tolerance by downregulating the reactivity of conventional CD25– CD4+ helper T-cells (32, 34). *Foxp3* is also expressed in lymphoid organs, such as spleen and thymus, where it plays an essential role during development, allowing the differentiation of the Treg population. The study of scurfy mice (mice with X-linked recessive mutation in *Foxp3*) revealed an overproliferation of activated CD4+ T-cells, resulting in dysregulation of lymphocyte activity (35-36). The lack of DNA binding domain of the protein leads to death of hemizygous males at 16-25 days after birth (35-36) and in the surviving mice in a great exacerbation of the autoimmune phenotype (37).

The corresponding human disorder is represented by Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX; also known as X-linked autoimmunity and allergic dysregulation

syndrome, XLAAD). This fatal recessive disorder is due to truncated protein or inhibition of DNA binding domain. It develops in early childhood and is associated with protracted diarrhoea, thyroiditis, dermatitis, allergic manifestations, insulin-dependent-type 1 diabetes and anaemia, besides massive T-cell infiltration into the skin and gastrointestinal tract and high serum levels of autoantibodies, as a sign of autoreaction. Recent studies have shown that the expression of FOXP3 and the subsequent conversion of human and mouse peripheral naïve T-cells in Treg is induced by TGF-β (38-39). Most probably, this event is mediated by activation of Small Mothers Against Decapentaplegic (SMAD) transcription factors. Generally, the inhibition of TGF-β-mediated signaling involves SMAD7 in an autoregulatory loop, but it was also shown that FOXP3 can inhibit it, as well. The induction of *FOXP3* expression by Treg results in a prolonged TGF-β-mediated signaling perhaps allowing the stabilization or expansion of the Treg pool (30).

FOXOs are the mammalian homologues of the *Caenorhabditis elegans* dauer formation mutant 16 (DAF-16) and, in this organism, they seem to be involved in longevity regulation. FOXO1 (FKHR, forkhead in rhabdomyosarcoma), FOXO3A (FKHRL1, FKHR-like 1), FOXO4 (AFX, mixed lineage-leukemia (trithorax homolog) translocated to 7 homolog, Mllt7) and FOXO644 are the most studied members of this family for their implication in the regulation of apoptosis, cell cycle, metabolism and resistance to oxidative stress (40-42). Gene targeting experiments in mice have demonstrated that FOXO1 regulates insulin sensitivity (43-44), adipocyte differentiation (44) and angiogenesis (45), while FOXO3A regulates ovarian development and fertility (46-48) and FOXO4 appears to be

largely dispensable for gross organismal homeostasis (48). FOXO proteins are ubiquitously expressed, even if there is a tissue specific expression for the diverse isoforms. While FOXO1 is ubiquitous, FOXO3A is expressed in lymphocytes and it appears the dominant isoform of the mammalian family. Cellular stimulation by mitogens or cellular stress, leads to activation of several intracellular kinases such as phosphatidyl inositol 3 kinase (PI3K), serum/glucocorticoid‐regulated kinase (SGK) and protein kinase B (PKB, Akt), resulting in the phosphorylation of the FOXOs. This makes FOXO unable to bind DNA and renders it susceptible to nuclear export and/or proteasome mediated degradation (IkB kinase (IKK)) thereby preventing FOXO‐mediated transcription (49-50). In resting cells, unphosphorylated forms of the FOXOs are localized in the nucleus, where they are transcriptionally active and regulate several biologic processes, including proliferation, apoptosis and response to cellular stress. To date, there is no evidence that an altered FOXO activity is associated with a human immunological disease. However, in mice a significant diminished FOXO activity in T‐cells is associated with autoimmune lupus syndrome, thus leading to hypothesize a possible relationship between the *FOXO* genes and inflammation in *humans* (50). Differently, *FOXO* gene dysregulation has been well documented in human cancer.

The FOXJ1 (hepatocyte nuclear factor/forkhead homolog‐4, HNF‐4, FKHL‐13) transcription factor plays an important role in the development of ciliated epithelia (51). Thus, FOXJ1 is expressed in all structures containing ciliated cells, such as the lungs, spermatids, oviducts and choroid plexus (52). The loss of FOXJ1 results in lethality in uterus or soon after birth as

demonstrated by observations of *Foxj1*–/– mice that die during embryonic development (53-54). In fact, *Foxj1*‐deficient mice are characterized by absence of cilia and, subsequently, suffer from significant developmental abnormalities including heterotaxy and hydrocephalus (30). Besides its role in the differentiation of ciliated cells, recently a new role for FOXJ1 has been discovered in the differentiation of other cell types. It was observed that *Foxj1* is downregulated in lymphocytes isolated from mice affected with systemic lupus erythematosus (SLE); this evidence suggests that FOXJ1 might prevent autoimmune reactions (55). FOXJ1 is expressed in naïve T‐cells and its downregulation occurs after IL‐2 and/or TCR stimulation (55). FOXJ1, similarly to FOXO3A, is required in vivo to modulate NF‐kB activity, upregulating IkBβ and maintains T‐cell tolerance, but unlike FOXO3A deficiency, FOXJ1 deficiency is much more severe, affecting a different spectrum of organs and Th1 cytokine production. Thus, these two forkhead members play either overlapping or clearly distinct roles in helper T‐cells, even though the intimate mechanisms remain to be elucidated (56).

Foxn1, a highly conserved transcription factor, has been previously extensively mentioned. It exerts its function after activation through phosphorylation that promotes its nuclear translocation (57-59). Into the nuclei it interacts with DNA as a monomer through its forkhead box, but the target genes and the specific biochemical mechanism of interaction with the promoter regions remain to be elucidated (28, 60). FOXN1 expression is strongly regulated by wingless (Wnt) proteins (61) and bone morphogenetic proteins (BMPs) (62) in both autocrine and paracrine fashions (56) and its expression is restricted to epithelial cells in the skin and in the thymus. There

are no data available on FOXN1 mRNA expression in liver, spleen, testis, lung, heart and brain, but murine choroid plexus (63). During embryogenesis, FOXN1 is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney and urinary tract. In adult life, its expression is limited to epithelial cells of the intestine, spermatocytes of the testis and thymus (64). In particular, on the basis of the observation that nude mice keratinocytes do not differentiate in a normal fashion, FOXN1 could be considered as a key regulator of the balance between keratinocytes growth and differentiation. It suppresses the involucrin and locrin expression, both components of the cornified envelope and the profilaggrin, involved in the aggregation of the intermediate filaments.

Other factors have been identified as Foxn1 target. In fact, recent studies have shown that Foxn1 is linked to Akt (PKB) expression (65), thus giving a possible explanation of FOXN1 involvement in epidermal layer regulation. FOXN1 also controls follicular formation, influencing the expression of two hair keratins, mHa3 and mHb5 (3).

All these findings associated with an extensive review of the literature have been the bases for a Book Chapter published by *Landes Bioscience*, that has received a great echo in the editorial and scientific communities and so it has been co-published by *Springer* and cited in Pubmed, for the chapter see below.

CHAPTER

Human Clinical Phenotype Associated with *FOXN1* Mutations

Claudio Pignata,* Anna Fusco and Stefania Amorosi

Abstract

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

Introduction: Severe Combined Immunodeficiencies

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

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

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

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

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

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

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

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

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

The Nude/SCID Phenotype

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

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

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

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

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

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

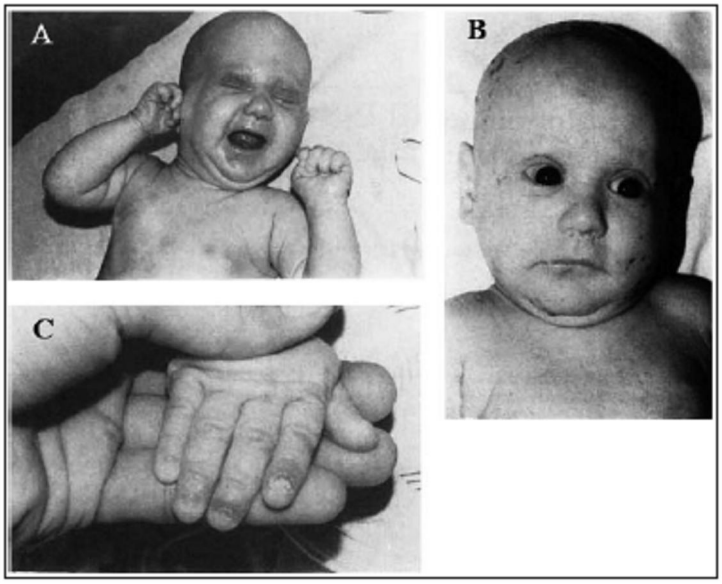

Figure 1. A,B) Alopecia of scalp, eyebrows and eyelashes in two sisters in whom the human Nude/SCID phenotype was first described. C) Nail dystrophy in human Nude/SCID. Reprinted with permission from: Pignata C, Fiore M, Guzzetta V et al. Congenital Alopecia and nail dystrophy associated with severe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

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

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

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

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

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

Reprinted with permission from: Pignata C. A lesson to unraveling complex aspects of novel immunodeficiencies from the human equivalent of the nude/SCID phenotype. J Hematother Stem Cell Res 2002; 11:409-414.

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

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

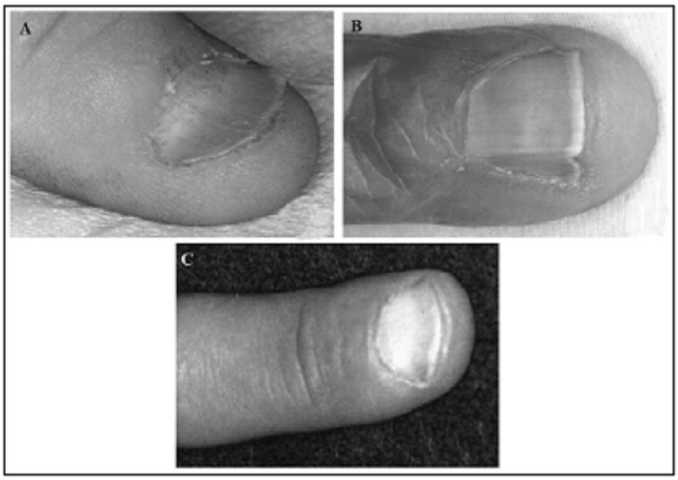

Figure 2. Nail dystrophy patterns in subjects heterozygous for the *FOXN1* mutation: A) koilonychia; B) canaliform dystrophy; and C) leukonychia. Reprinted with permission from: Auricchio L, Adriani M, Frank J et al. Nail distrophy associated with a heterozygous mutation of the Nude/SCID human *FOXN1*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

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

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

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

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

Fox Family Members and Immune System

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

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

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

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

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

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

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

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

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

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

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

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

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

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

FOXN1 Skin Specific Expression and T-Cell Development

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

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

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

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

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

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

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

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

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Conclusive remarks

The cited work contains recent information that came out from the original description of Nude/SCID phenotype. For the first time, only recently, a careful description of clinical manifestations associated with an alteration of the FOXN1 gene has been provided, thus leading to identify the human equivalent of the well studied spontaneous murine Nude/SCID immunodeficiency. In this context, alterations of FOXN1 and of other members of FOX subfamilies are now emerging as intriguing causes of immunological disorders mainly characterized by abnormal T‐cell development or abnormal T‐cell regulatory homeostasis. Eventually, it should be underlined that the Nude/SCID phenotype is the only form of SCID associated with an alteration of a gene that is not expressed in the hematopoietic cell.

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

§ 2.2 FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus

In the village where the first Nude/SCID patients were identified a genetic counselling was offered to couples at risk. The prenatal diagnosis by direct genetic analysis of *FOXN1* led to identify an affected female fetus during the first trimester of pregnancy, thus indicating the importance of this effort. The fetus was homozygous for R255X mutation and the autoptical examination revealed the absence of the thymus and a grossly abnormal skin which was tighter than usual and which showed basal hyperplasia and dysmaturity, suggestive of an impaired differentiation program. These signs confirmed that FOXN1 is involved in thymic development and skin differentiation in humans. Of note, the fetus also showed multiple-site neural tube defects, including anencephaly and spina bifida that could explain the high rate of mortality in uterus observed in the described population. During our study, we also showed that while there have been no prior studies of FOXN1 activity in the nervous system, we observed that the mouse *Foxn1* gene is expressed in epithelial cells of the developing choroid plexus, a structure filling the lateral, third, and fourth ventricles of the embryonic brain.

Another aspect that led to consider the Nude/SCID mutation and anencephaly causally related is the fact that the other forms of SCID become clinically evident in post-natal life, when the protection of the newborn by the mother immune system declines.

Moreover, the expression of Foxn1 in the embryonic choroid plexus is consistent with a possible role for Foxn1 in the development of the central nervous system. The expression in choroid plexus is intriguing, suggesting

that disturbances in cerebrospinal fluid (CSF) secretion may play a role in the pathogenesis of defects at both ends of the neural tube.

After this first observation, we identified a second fetus belonging to the same family that carried the R255X homozygous mutation in *FOXN1*. This fetus was examined for the presence of central nervous system (CNS) developmental anomalies. Prenatal diagnosis, performed by villocentesis at 11 weeks of gestation, led to a diagnosis of Nude/SCID syndrome. At 16 weeks of gestation, the abdominal sonography of the Nude/SCID fetus revealed a morphologically normal brain, but with absence of cavum septum pellucidum (CSP), in that only medial wall of the frontal horn was observed. At this age, CSP is a fluid-filled cavity situated between the membranes which form the septum pellucidum and bounded superiorly and anteriorly by corpus callosum and inferiorly by the fornix and is considered a marker of a normal anatomy (66). However, this structure is seen in the 40% of cases at 15 weeks, 82% at 16-17 weeks, and 100% after the 18th week of gestation (66). After parents decision, the termination of pregnancy was performed at 18th week and the fetus was further examined postmortem after obtaining informed consent. At autopsy, the brain weight was 35 g and a first gross examination revealed an enlargement of the interhemispheric fissure. A magnetic resonance imaging (MRI) of the extracted brain 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. Convexity sulci and lamination of brain parenchyma were normal for gestational age (67-68). On the contrary, corpus callosum could not be identified in any section. Furthermore, in keeping with this, subsequent autopsy failed to reveal any macroscopic

abnormality of brain structures, but the abnormality in the development of corpus callosum.

The first of these 2 observations has been published on *Clinical Genetics*, while the other one has been submitted to the *Journal of Neurology* and it is under review, for the manuscripts see below.

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Short Report

FOXN1 homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus

Amorosi S, D'Armiento M, Calcagno G, Russo I, Adriani M, Christiano AM, Weiner L, Brissette JL, Pignata C. *FOXN1* homozygous mutation associated with anencephaly and severe neural tube defect in human athymic Nude/SCID fetus.
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The forkhead, Fox, gene family comprises a diverse group of 'winged-helix' transcription factors that play important roles in development, metabolism, cancer and aging. Recently, several forkhead genes have been demonstrated to play critical roles in lymphocyte development and effector functions. Alterations of the *FOXN1* gene in both mice and humans result in a severe combined immunodeficiency caused by an intrinsic defect of the thymus associated with congenital alopecia (Nude/severe combined immunodeficiency phenotype). FOXN1 is a member of the class of proteins involved in the development and differentiation of the central nervous system. We identified a human fetus homozygous for a mutation in *FOXN1* gene who lacked the thymus and also had abnormal skin, anencephaly and spina bifida. Moreover, we found that *FOXN1* gene is expressed in mouse developing choroid plexus. These observations suggest that FOXN1 may be involved in neurulation in humans.

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Key words: anencephaly – FOXN1 – neural tube defects – SCID – thymic aplasia

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The forkhead (Fox) gene family comprises a large and diverse group of transcription factors that share a 'winged-helix' DNA-binding domain, consisting of three alpha helices flanked by two 'wings' of beta strands and loops (1–3). The name forkhead is derived from the *Drosophila melanogaster forkhead* (*fkh*) gene product, which is required for terminal pattern formation of the embryo (4). The increase in complexity of the organisms with evolution has provided the driving force for the expansion of this family of proteins. Over 100 proteins with forkhead domains grouped in at least 17 subclasses, FoxA through FoxQ, have been found (5). All of them play critical roles in multiple biological processes, including development, metabolism, aging and

cancer (6). The forkhead family is important in patterning the midline of the neural tube and is expressed as early as the neural placode.

FOX gene mutations have been found to be responsible for diverse phenotypes, ranging from alterations of craniopharyngeal development (*FOXE1*) to speech and language abnormalities (*FOXP2*) or hearing defects in mice (*Foxi1*) (7). Foxn1 (hepatocyte nuclear factor/forkhead homolog-11, Hfh-11, FKHL20, Whn, nude, nu) is implicated in cutaneous and thymic epithelial cell development (8, 9). It is best known immunologically for its association with the mouse and human *nude* (*nu*) mutations, which result in severe combined immunodeficiency (SCID) syndrome associated with congenital alopecia and nail dystrophy (Nude/SCID phenotype; MIM 601705) (10, 11).

SCIDs are disorders of both cell-mediated and humoral immunity, characterized by high susceptibility to develop severe and sometimes fatal infections (12). These syndromes do not affect pre-natal life because of the maternal protection and, usually, become clinically evident by the first months of post-natal life. In most cases, these diseases are caused by molecular alterations of genes expressed in hematopoietic cells. Different from the other forms, this novel form of SCID is caused by an intrinsic defect of the thymus. Recently, we carried out a genetic screening of the village population from which the two patients with Nude/SCID phenotype originated, which led to the identification of 55 heterozygous subjects for the *FOXN1* mutation (13).

Here, we report on a female human fetus who was homozygous for the R255X mutation of the *FOXN1* gene and had a severe neural tube defect.

Materials and methods

Genetic counseling

During the past 5 years, a genetic counseling program was offered to the village population from which the two Nude/SCID patients originated. Because *FOXN1* mutation is particularly devastating as it leads to the absence of the thymus and SCID, pre-natal diagnosis by direct genetic analysis was performed for couples at risk. The genetic counseling also included taking a detailed family history and drawing a family tree.

Mutational analysis

After written informed consent was obtained from parents and upon approval of the Institutional Ethical Committee, genomic DNA was extracted following standard procedures from amniocytes and processed anonymously (14). This procedure was performed in a laboratory with 'Clinical Laboratory Improvement Amendments certification', and *FOXN1* DNA analysis was performed according to a polymerase chain reaction (PCR) assay previously described (13). Briefly, a PCR fragment containing exon 5 of the *FOXN1* gene was amplified using the primers exon 5F: 5′-CTTCTGGAGCGCAGGTTGTC-3′ and exon 5R: 5′-TAAATGAAGCTCCCTCTGGC-3′. Aliquot of the samples to be studied were digested with the restriction enzyme *Bsr*I and the product was electrophoresed on 1.5% agarose gel. PCR fragments positive to the digestion with *Bsr*I were then purified on Edge Centriflex columns (Edge BioSystems, Gaithersburd, MD) and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer from Applied Biosystems (Perkin Elmer, Waltham, MA). After informed consent, fetus underwent physical examination by our pathologist in order to define whether *FOXN1* homozygous alteration was associated with defects in the differentiation process and fetus development.

Results

Genetic counseling and identification of the Nude/SCID fetus

During genetic counseling, a female fetus homozygous for the R255X mutation of the FOXN1 product was identified at 15 weeks of gestation. The *FOXN1* mutation was a C792T transition in exon 5 upstream of the DNA binding and transactivation domain and is the same as that previously described by Frank et al. (11). This led to termination of pregnancy.

Pathologic examination

Identical to the Nude/SCID phenotype of mice, this fetus lacked a thymus (Fig. 1a), confirming that *FOXN1* is essential for thymic development in humans. The skin appeared grossly abnormal, being tighter than usual and showing basal hyperplasia and dysmaturity, suggestive of an impaired differentiation program. In addition, the fetus had multiple-site neural tube defects, including anencephaly and spina bifida (Fig. 1 b,c). While there have been no prior studies of *FOXN1* activity in the nervous system, we observed that the mouse *Foxn1* gene is expressed in epithelial cells of the developing choroid plexus, a structure filling the lateral, third, and fourth ventricles of the embryonic brain (Fig. 2).

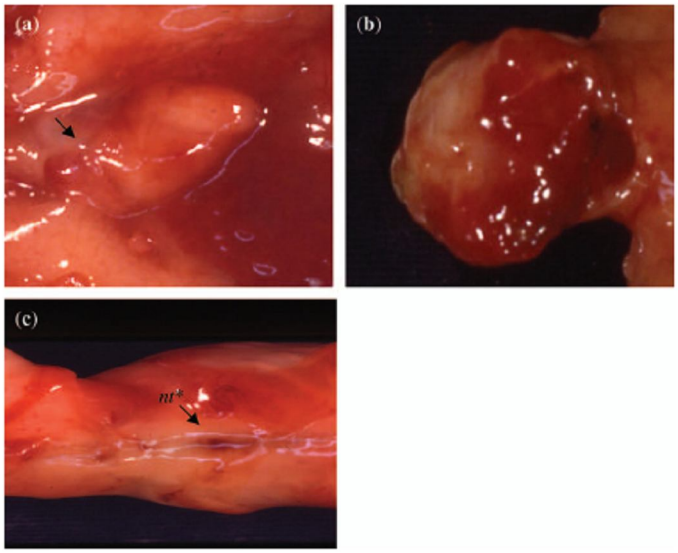

Fig. 1. Morphological phenotype of FOXN1$^{-/-}$ human. Pregnancy was interrupted at 15 weeks. The skin was tight, shiny and smooth. (a) Ventral view of the fetus showing the absence of thymus in the chest. The arrow indicates the usual thymus location. (b) Dorsal view showing the skull of the FOXN1$^{-/-}$ fetus. Complete anencephaly characterized by the absence of scalp, calvarium and brain. (c) Dorsal view showing the craniospinal rachischisis. nt*, open neural tube (spina bifida).

Discussion

These observations suggest that *FOXN1* may be involved in brain and neural tube development and function in a similar fashion to other members of the forkhead/winged-helix class proteins, such as mouse *HNF-3β* and *BF-1* (15) and human *FOXP2* (16). Mice defective in *HNF-3β* through gene targeting technology lack a notochord, which results in severe defects of neural tube organization (15). In addition, other members of the FOX family, such as Foxj1, have already been identified as important molecules for development of choroid plexus epithelium, thus implying a potential role for these proteins in the nervous system development. It has been recently observed that in *Xenopus laevis*, another FOXN factor, FOXN4, is expressed in brain and retina at the earliest stages of maturation. FOXN4 is also expressed in the pallium, optic tectum, isthmus, reticular formation, and in cells lining the ventricle of the tadpole brain (17). In humans, alterations in the *FOXP2* gene are associated with neurodevelopmental disorders, suggesting that the molecule has a key role in neural functioning (16). Thus, different from Foxp2 that is expressed later as a regulator gene, Foxnl seems to be implicated as an organizer in neurulation.

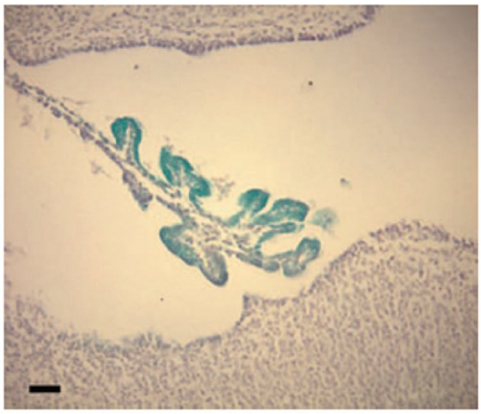

Fig. 2. Foxn1 expression in the brain murine embryos. A β-galactosidase reporter gene was inserted into the Foxn1 locus by homologous recombination (27). Heterozygous embryos (Foxn1^{+}/Foxn1^{lacZ}), which are phenotypically normal, were stained for β-galactosidase activity (blue) as described (28). Foxn1 is expressed throughout the epithelium of the developing choroid plexus (scale bar represents 50 μm).

Furthermore, as the choroid plexus modulates the composition of the cerebrospinal fluid (CSF), transporting or secreting key molecules into the brain's environment, it is thought that plexus cells have profound effects on the morphogenesis of neural structures (18). Thus, the expression of *Foxn1* in the embryonic choroid plexus is consistent with a possible role for *Foxn1* in the development of the central nervous system. The expression in choroid plexus is intriguing, suggesting that disturbances in CSF secretion may

play a role in the pathogenesis of defects at both ends of the neural tube. As previously described, the mechanism of delay or failure of neuroepithelial curvature that leads to neural tube defects in curly tail embryos involves abnormalities of neuroepithelial–mesenchymal interactions that may be initiated by abnormal cellular function within the neuroepithelium. So, it is not surprising that the absence of a factor expressed in choroid plexus could lead to neural tube defects (19). In addition, choroid plexus epithelium is a specialized ependyma whose secretory proteins are important in several developmental processes. Alterations of its immunocytochemical profile have also been found in certain cerebral malformations (20). A prominent feature of human anencephaly is the development of the area cerebrovasculosa, a fibrovascular proliferative phenomenon generally attributed to an encephaloclastic response of the toxic effects of chronic direct exposure of neural tissue to amniotic fluid. The *FOXN1* genetic mutation could play a role in the development of this unique histopathological feature. A possible explanation of the reported features is that an impaired ectodermal development with a consequent defective epidermis overlying the neural folds differentiation may have prevented the neural folds from coming together to form the neural tube causing anencephaly and spina bifida.

How FOXN1 regulates its functions is largely unknown. There have been relatively few studies on its molecular pathways and its own targets. Recently, it has been shown that phospholipase C-delta 1 is an essential molecule downstream of Foxn1 in normal hair formation, suggesting a possible explanation of hairlessness in nude mice caused by insufficient expression of this molecule (21).

There are a few considerations that suggest that the association between the Nude/SCID mutation and anencephaly may be causally related. First of all, other forms of SCID become clinically evident only after the first 3–4 months of post-natal life, when the protection of the newborn by the mother immune system declines. Overall, congenital SCIDs are not related to increased spontaneous interruption of pregnancy. In the community where the human Nude/SCID was discovered, there is a high rate of abortions in the first trimester in the marriages between subjects heterozygous for the *FOXN1* mutation. This finding suggests that FOXN1 may also be implicated in the development because the first trimester mortality is not justified by the SCID *per se*. Although there is no demonstration that there are gross brain malformations in mice, a reduced number of Purkinje cells of the cerebellum has been reported in Nude/SCID mice, which is in addition reduced in size (22). Furthermore, perinatal death was reported in the spontaneous strain 'nude Yurlovo' (23). It is to note that organogenesis in mice lasts throughout the pregnancy, while in humans, it mainly occurs in the first 3 months. The association herein reported in man, indicating that the human phenotype is more severe than in mice, could explain the reason as to why the human counterpart of the mouse Nude/SCID, described in 1966, has only been identified 30 years later (10). As for the variability of clinical expression of the same gene mutation, this is not unusual in neurodevelopmental disorders (24, 25). A similar observation has been reported in lathosterolosis where the same enzymatic alteration led to multiple organ malformations in one living sibling and severe neural tube defects in a fetus from the same parents (26). A possible explanation is that a more severe clinical expression of the Nude/SCID may lead to pre-natal lethality and a milder one to a newborn with Nude/SCID.

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Title: Brain development in a Nude/SCID fetus carrying FOXN1 homozygous mutation Column Title: Brain development in Nude/SCID

Article Type: Letter to the Editor

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Abstract: 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, who 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 weeks of gestation, the abdominal ultrasonography of the Nude/SCID fetus revealed a morphologically normal brain, but with absence of cavum septum pellucidum (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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Brain development in a Nude/SCID fetus carrying *FOXN1* homozygous mutation

Column Title: Brain development in Nude/SCID

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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, who 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 weeks of gestation, the abdominal ultrasonography of the Nude/SCID fetus revealed a morphologically normal brain, but with absence of cavum septum pellucidum (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.

Key words brain development · FOXN1 · corpus callosum · SCID · thymic aplasia

Dear Sir

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 due to a profound T-cell defect [3].

Recently, our group reported on a human fetus affected with Nude/SCID phenotype due to *FOXN1* gene mutation who also had severe neural tube defects, namely anencephaly and spina bifida. The affected fetus was identified during a prenatal genetic counselling program offered to couples at-risk in a village. 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.

Case Report

We report on a second fetus belonging to the same family, that carried the R255X homozygous mutation in *FOXN1,* examined for the presence of CNS developmental anomalies. Prenatal diagnosis, performed by villocentesis at 11 weeks of gestation, led to a diagnosis of Nude/SCID syndrome. At 16 weeks of gestation, the abdominal sonography of the Nude/SCID fetus revealed a morphologically normal brain, but with absence of cavum septum pellucidum (CSP), in that only medial wall of the frontal horn was observed (Fig. 1a). At this age, CSP is a fluid-filled cavity situated between the membranes which form the septum pellucidum and bounded superiorly and anteriorly by corpus callosum and inferiorly by the fornix and is considered a marker of a normal anatomy [5]. However, this structure (Fig. 1b) is seen in the 40% of cases at 15 weeks, 82% at 16-17 weeks, 100% after the 18th week of gestation [5]. After parents decision, the termination of pregnancy was performed at 18th week and the fetus was further examined postmortem after obtaining informed consent. At autopsy, the brain weight was 35 g and a first gross examination revealed an enlargement of the interhemispheric fissure. A magnetic resonance imaging (MRI) of the extracted brain 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. Convexity sulci and lamination of brain parenchyma were normal for gestational age [6, 7]. On the contrary, corpus callosum could not be identified in any section (Fig. 2a-e). Furthermore, in keeping with this, subsequent autopsy failed to reveal any macroscopic abnormality of brain structures, but the abnormality in the development of corpus callosum (Fig. 2f).

Discussion

In conclusion, our observations 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. However, the presence of an alteration 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 observed in the developing eye and several other brain structures [10]. In addition, we previously found that *FOXN1* gene is expressed in mouse developing choroid plexus. Recently, it has also been shown that Foxn1 is required to maintain the expression in the hair

follicle matrix of Notch1 [11], whose 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 development abnormalities.

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Legend to figures

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, d Axial (cerebellum, ganglionic eminence, mesencephalon and dorsal fronto-parietal cortex) and **e** coronal MRI sections showing the integrity of major structures, but corpus callosum could not be identified in any section. **f** Coronal view of the brain at autopsy.

Figure 1
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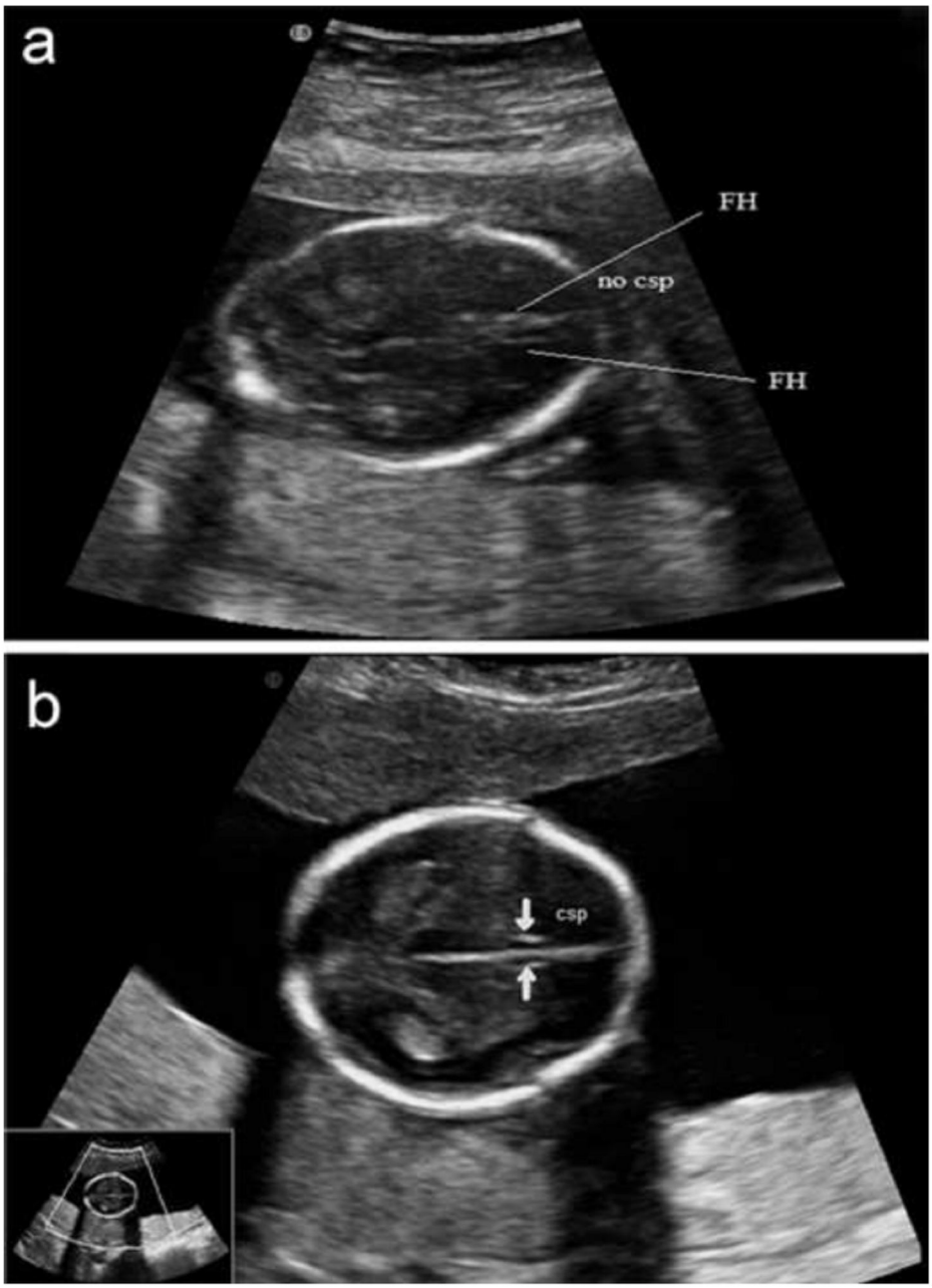

Figure 2
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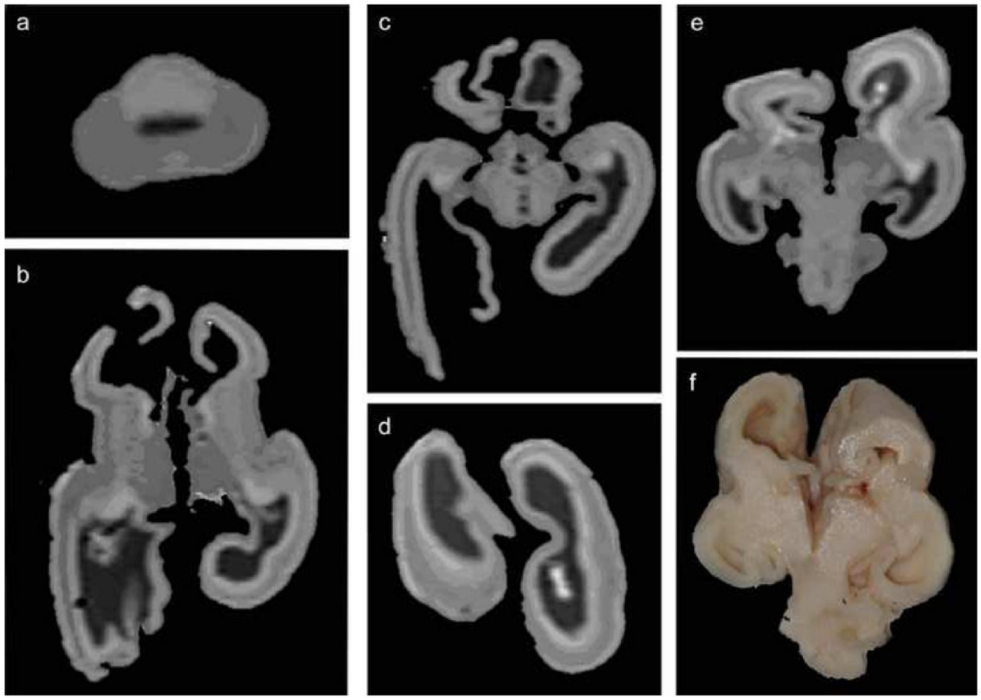

Conclusive remarks

The reported observations open a new window in the comprehension of the role of FOXN1 in the development of different tissues, such as CNS, confirming former observations related to other FOX family members (29). Although our reports revealed 2 distinct CNS phenotypes associated to the same *FOXN1* gene mutation (R255X), they would suggest that *FOXN1* alterations could play a role as 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 (69-70).

It should be noted that mice defective in HNF-3b through gene targeting technology lack a notochord, which results in severe defects of neural tube organization (71). In addition, other members of the FOX family, such as Foxj1, have already been identified as important molecules for development of choroid plexus epithelium, thus implying a potential role for these proteins in the nervous system development. It has been recently observed that in Xenopus laevis, another FOXN factor, FOXN4, is expressed in brain and retina at the earliest stages of maturation. FOXN4 is also expressed in the pallium, optic tectum, isthmus, reticular formation, and in cells lining the ventricle of the tadpole brain (72). In humans, alterations in the FOXP2 gene are associated with neurodevelopmental disorders, suggesting that the molecule has a key role in neural functioning (73). Thus, different from Foxp2 that is expressed later as a regulator gene, Foxn1 seems to be implicated as an organizer in neurulation. Moreover, the zebrafish orthologue of the mouse nude gene *Foxn1* is observed in the developing eye and several other brain structures (74). Recently, it has also been shown that Foxn1 is

required to maintain the expression in the hair follicle matrix of Notch1 (75), whose signaling is known to regulate cell fate specification and pattern formation in the developing nervous system (76).

Nevertheless, the precise role of the FOXN1 transcription factor in CNS development remains to be fully clarified. Our cases should encourage physicians and pathologists to search for FOXN1 alterations in congenital brain development abnormalities.

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CHAPTER 3

The Ataxia Telangiectasia

Among those PIDs whose causing genes are not selectively expressed in the hematopoietic compartment there is Ataxia-telangiectasia (A-T), a rare autosomal recessive disorder, characterized by progressive neurological dysfunction, especially affecting the cerebellum, oculo cutaneous telangiectasia, high incidence of neoplasms and hypersensitivity to ionizing radiations (1-2).

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 (3). The immunodeficiency is progressive and the most consistent immunodeficiency reported is poor antibody response to pneumococcal polysaccharide vaccines (3). Moreover, serum concentration of the immunoglobulins IgA, IgE and IgG2 may be reduced. Approximately 30% of individuals with A-T who have immunodeficiency have T-cell deficiencies. At autopsy, virtually all individuals have a small embryonic-like thymus. Unlike most immunodeficiency disorders, the spectrum of infection in individuals with A-T does not comprise opportunistic infections. Some individuals develop chronic bronchiectasis and the frequency and severity of infections correlates more with general nutritional status than with the immune status. Individuals with frequent and severe infections appear to benefit from intravenous immunoglobulin (IVIG) replacement therapy (3); however, longevity has increased substantially even in individuals not receiving IVIG.

The disease is associated with mutations in the ataxia telangiectasia mutated (ATM) gene encoding for a 370 kDa serine/threonine kinase, that shares sequence similarities with the catalytic subunit of phosphatidyl-inositol-3-kinase (4). Most of its substrates appear to be involved in cell cycle checkpoints and DNA double-strand break (DSB) response (5). Thus, the disease is considered the prototype of the DNA-repair defect syndromes (6).

The clinical course of the disease is severe, and the median survival of A-T patients is actually 19–25 years with a wide range (7). A variable immune defect has been long recognized in patients with A-T (8). Immunological abnormalities mainly include impaired antibody responses, T-cell lymphopenia, primarily affecting CD4+ lymphocytes, and impaired proliferative response to mitogens and antigens (3). However, severe infections are uncommon in A-T (3). On the contrary, clinical signs related to the progressive neurodegeneration are overwhelming and dramatically affect the quality of life. Purkinje cells are thought to be selectively depleted, then resulting in the progressive cerebellar atrophy of the cortex associated with significant thinning of the molecular layer, as revealed by autopsy and biopsy studies (9-10). 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

A recent clinical observation reported on a dramatic improvement of neurological symptoms following a short course of oral betamethasone in a child affected with A-T (11). Due to the importance of the topic, this study aimed to extend such an observation to additional A-T patients referred to a single Immunodeficiency Center.

In this part of the study, we reported on the amelioration of neurological signs, assessed by Scale for the Assessment and Rating of Ataxia (SARA), during short-term treatment with oral betamethasone. Of note, this effect was observed not only in very young A-T patients, but also in older ones. The clinical response was observed as soon as 48 h following the beginning of the therapy and was transient, in that it disappeared soon after therapy withdrawal. The highest clinical improvement was on speech disturbance, stance, and finger chase items.

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Steroid-induced improvement of neurological signs in ataxia-telangiectasia patients

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ataxia-teleangiectasia, betamethasone

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A recent clinical observation reported on a dramatic improvement of neurological symptoms following short-term betamethasone administration in a child affected with ataxia-teleangiectasia (A-T). The aim of this study was to extend this observation to additional A-T patients followed at a single Immunodeficiency Center. Six consecutive patients (three males; mean age 16.3 years, range 5–30 years) were enrolled into this monocentric before–after trial. A cycle of oral betamethasone at the dosage of 0.1 mg/kg/day was administered for 10 days. The neurological evaluation was performed through the Scale for the Assessment and Rating of Ataxia. Overall, five of the six patients exhibited a clear amelioration of the neurological performances. Only in two patients, a slight amelioration persisted 7 days after the therapy withdrawal, whilst in the other patients the score reached approximately the pre-treatment value at the end of the therapy. Twenty-eight of the 46 evaluated neurological items (60%) improved during therapy. The speech disturbance, finger chase and nose–finger test showed the more significant improvement. The clinical amelioration was inversely correlated with the level of cerebellum atrophy, as revealed by the magnetic resonance. Our data indicate that neurological signs in A-T are susceptible of beneficial pharmacological intervention even years after the disease onset.

Introduction

Ataxia-telangiectasia (A-T) is a rare autosomal recessive disorder, characterized by progressive neurological dysfunction, especially affecting the cerebellum, oculocutaneous telangiectasia, immunodeficiency, high incidence of neoplasms and hypersensitivity to ionising radiations [1,2]. The disease is associated with mutations in the ataxia telangiectasia mutated (ATM) gene encoding for a 370 kDa serine/threonine kinase, that shares sequence similarities with the catalytic subunit of phosphatidyl-inositol-3-kinase [3]. Most of its substrates appear to be involved in cell cycle checkpoints and DNA double-strand break response [4]. Thus, the disease is considered the prototype of the DNA-repair defect syndromes [5].

The clinical course of the disease is severe, and the median survival of A-T patients is actually 19–25 years with a wide range [6]. A variable immune defect has been long recognized in patients with A-T [7]. Immunological abnormalities mainly include impaired antibody responses, T-cell lymphopenia, primarily affecting CD4+ lymphocytes, and impaired proliferative response to mitogens and antigens [8]. However, severe infections are uncommon in A-T [8]. On the contrary, clinical signs related to the progressive neurodegeneration are overwhelming and dramatically affect the quality of life. Purkinje cells are thought to be selectively depleted, then resulting in the progressive cerebellar atrophy of the cortex associated with significant thinning of the molecular layer, as revealed by autopsy and biopsy studies [9,10].

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.

A recent clinical observation reported on a dramatic improvement of neurological symptoms following a short course of oral betamethasone in a child affected with A-T [11]. Due to the importance of the topic, the aim of this study was to extend such an observation to additional A-T patients referred to a single Immunodeficiency Center.

Subjects and methods

Six consecutive patients (three males) of 5, 6, 14, 15, 27 and 29 years of age, respectively (mean age 16.3 years, range 5–29 years), affected with A-T were enrolled in this monocentric study. The patients received a

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

	P1	P2	P3	P4	P5	P6
Sex	F	F	M	F	M	M
Age (years)	29	27	14	15	5	6
Age at onset of unsteadiness of gait (years)	6	6	2	2	2	2
Loss of ambulatory skills (years)	Still ambulant	Still ambulant	9	9	Still ambulant	Still ambulant
Cerebellar ataxia	+ + +	+ + +	+ + +	+ + +	+ +	+ +
Resting tremor	+ + +	+ + +	+ + +	+ + +	±	±
Increased AFP levels	Present	Present	Present	Present	Present	Present
ATM mutation	3576 G > A	3576 G > A	1463 G > A	NA	c.97delC c.2113delT	717delCCTC

A-T, ataxia telangiectasia; NA, data not available; AFP, alpha fetoprotein; ±, borderline; + +, moderate; + + +, severe.

diagnosis of A-T according to the European Society of Immunodeficiencies (ESID) criteria. Table 1 shows that the four patients (P3, P4, P5, P6) had an early onset of symptoms, whilst two a later onset. A previous molecular study revealed a mutation in all patients and absence of the protein, but in P4, in whom the mutation was not yet identified, even though the ATM gene was not completely sequenced. However, the ATM protein level in this patient was approximately 20% of controls, indicating that the pathogenesis also involves ATM protein [12]. Brain MRI was performed at 1.5 tesla. The neurological evaluation was performed through the Scale for the Assessment and Rating of Ataxia (SARA) [13]. This scale was selected both to ensure specificity for ataxia and to utilize standardized procedures. The scale consists of eight items that yield a total score of 0 (no ataxia) to 40 (most severe ataxia). These items include the following: gait, sitting, heel-shin slide, stance, finger chase, nose–finger test, fast alternating hand movements and speech disturbance. Limb kinetic functions (items 5–8) are rated independently for both sides, and the arithmetic mean of both sides is included in the SARA total score (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 two pediatric neurologists. A third physician reviewed the videotape blinding, thus without knowing whether that specific neurological evaluation was recorded before, during or after the treatment. The final results were obtained through the comparison of the individual evaluator's scores.

Immunological studies included the evaluation of serum immunoglobulin levels and of the *in vitro* proliferative response to mitogens with standard procedures. Phytohemagglutinin (PHA), phorbol myristate acetate (PMA) and poke weed 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.

The patients, upon written consent, received a cycle of oral betamethasone at the dosage of 0.1 mg/kg/day for 10 days divided every 12 h. The clinical and laboratory evaluations were carried out before therapy (T0), 48 h from the beginning (T48), at the end of the therapy (T10d) and, eventually, 7 days from the withdrawal (T + 7). The adverse effects were monitored through routine laboratory tests, including blood cell count, serum levels of transaminases, plasma electrolyte levels, and renal functionality. Furthermore, weight and blood pressure were measured. The protocol was formally approved by the local Ethical Committee.

Results

At the beginning of the study, the neurological evaluation in our A-T patients revealed severe cerebellar ataxia and resting tremor in four patients (P1, P2, P3, P4) and moderate neurological alterations in the remaining. Four patients could walk, either autonomously (P5, P6) or with support (P1, P2). All patients showed instability of gait, posture, seating and head, diminished postural tone, dysmetria, and dysarthria. Cognitive status was normal in all patients. Table 2 summarizes the total SARA score in each patient. Overall, five of the six patients exhibited a clear amelioration of the neurological performances. In one patient, (P6) the improvement persisted 7 days after the therapy withdrawal, whilst in the remaining patients,

Table 2 Total SARA score in A-T patients

Time	P1	P2	P3	P4	P5	P6
T0	25	32	28	26	17	16
T48	27	30	27	24	14	6
T10d	28	27	24	21	11	9
T + 7	27	30	27	25	17	11

A-T, ataxia telangiectasia; SARA, Scale for the Assessment and Rating of Ataxia; T0, before therapy; T48, 48 h from the beginning of therapy; T10d, end of therapy; T + 7, off therapy.

Table 3 Changes of individual items of the SARA score during steroid therapy in A-T patients

	P1		P2		P3		P4		P5		P6	
	CCS−	CCS+	CCS−	CCS+	CCS−	CCS+	CCS−	CCS+	CCS−	CCS+	CCS−	CCS+
Gait	6	6	NA	NA	6	6	6	6	2	1	2	1
Sitting	1	2	1	1	1	1	2	2	1	0	1	0
Heel shin slide	1	1	NA	NA	1	1	2	1	1	1	1	0
Stance	5	5	6	5	6	5	5	3	2	1	1	0
Finger chase	2	2	4	3	3	2	3	2	3	1	2	0
Nose finger	3	3	3	1	2	1	2	2	1	1	1	0
Fast alterning hand movements	3	3	3	3	3	3	4	3	3	2	4	3
Speech disturbance	4	4	4	2	4	2	4	2	4	2	4	1

A-T, ataxia telangiectasia; NA, data not available; SARA, Scale for the Assessment and Rating of Ataxia; CCS−, before steroid therapy; CCS+, during steroid therapy.

the score reached approximately the pre-treatment value at the end of the therapy. Table 3 shows the changes of the SARA score for each item on an individual basis. In our study, we totally evaluated 46 parameters. Eight items were evaluated for each patient, except in P2 in whom items 1 and 3 could not be evaluated because of an accidental tibia and fibula fracture of the left leg treated by Hoffman external fixation. However, the assessment was carried out under parents' request. We observed that 28/46 variables (60%) improved during steroid therapy. In particular, three items showed the most significant improvement. Speech disturbance improved in all patients, occurring as soon as 48 h after the beginning of therapy, and persisted all over the trial. However, the most significant improvement was observed at T10d. Stance and finger chase tests ameliorated in five patients at different time. Stance ameliorated in four of the five patients (P2, P3, P4, P6) at T48 and at T10d in the other one (P5). Finger chase test ameliorated in three patients (P2, P3, P4) at T10d and in the others (P5, P6) at T48.

Brain MRI revealed no cerebellar abnormalities in P5 and P6. In the remaining patients the following patterns of cerebellar atrophy were detected: moderate cerebellar atrophy with a more marked atrophy of the vermis than cerebellar hemispheres in P3 and P4, and severe and marked diffuse atrophy of vermis and hemispheres in P1 and P2 (Fig. 1). When the results of the neurological improvement during the steroid therapy were compared with the pattern of cerebellar atrophy at MRI, an inverse correlation between the degree of cerebellar atrophy and the extent of amelioration was found.

Lymphocytes count was evaluated at the different times (Fig. 2). In five of the six patients, the number of lymphocytes increased, in four by T10 and in one patient the increase occurred at T + 7 (P6). However, only in two patients the increase in the cell count was remarkable (1170 vs. 1960 cells/mm^3, 1990 vs. 3350 cells/mm^3).

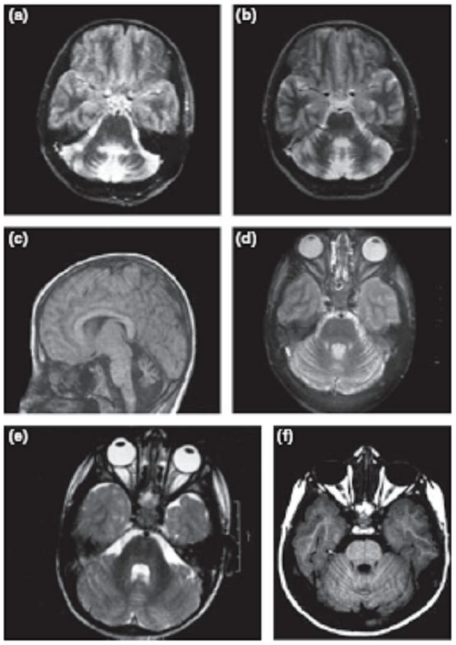

Figure 1 Brain structural MRI in six ataxia-teleangiectasia (A-T) patients. (a, b) T2 sequence on axial sections showing marked cerebellar atrophy in P1 and P2. (c) T1-weighed sagittal section showing moderate cerebellar and vermian atrophy. (d) T2 sequence on axial sections showing moderate cerebellar and vermian atrophy. (e, f) Normal cerebellum in P5 and P6.

Moreover, the proliferative response to mitogens was evaluated at the same time points to define whether the increase in lymphocyte count paralleled an increase in the cell proliferation. In most cases, the proliferative

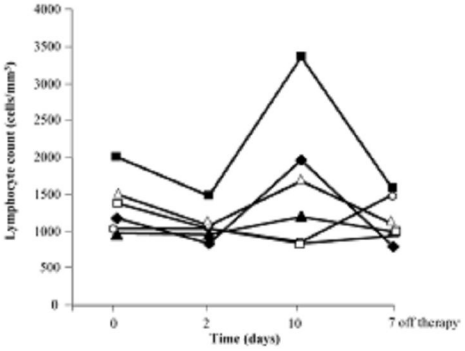

Figure 2 Lymphocyte count in six ataxia-teleangiectasia (A-T) patients. Graph showing lymphocyte count, expressed as cells/mm^3, in the patients before, during and 7 days off therapy.

response to the common mitogens, PHA, PMA, PWM, decreased by 7–97% after 48 h from the beginning of the therapy (Fig. 3). However, in four of the six patients, the proliferative response to PHA increased later on during the therapy, reaching the pre-treatment value. Of note, in two cases the post-treatment value was higher than the T0 value (36060 vs. 26486 cpm and 12866 vs. 3157 cpm, respectively). The proliferative response to PMA or PWM was similar to the PHA response.

Discussion

Ataxia-teleangiectasia is primarily a neurodegenerative disorder, whose underlying pathology consists of a progressive cerebellar degeneration, mainly involving Purkinje and granule cells, associated with immunodeficiency [14]. In this study, we report on the amelioration of neurological signs during short-term treatment with oral betamethasone. Of note, this effect was observed not only in very young A-T patients, but also in older ones. The clinical response was observed as soon as 48 h following the beginning of the therapy and was transient, in that it disappeared soon after therapy withdrawal.

The clinical phenotype of A-T is quite heterogeneous with a surprising, and still unexplained, different impact of the pathogenic mechanism on the severity of immunological and neurological signs. Differently from lymphocytes, whose turn-over is continuous and characterized by the release into the peripheral stream of fully differentiated mature cells from bone marrow, the placement of Purkinje neurons occurs relatively early in human development [1]. This would suggest that early

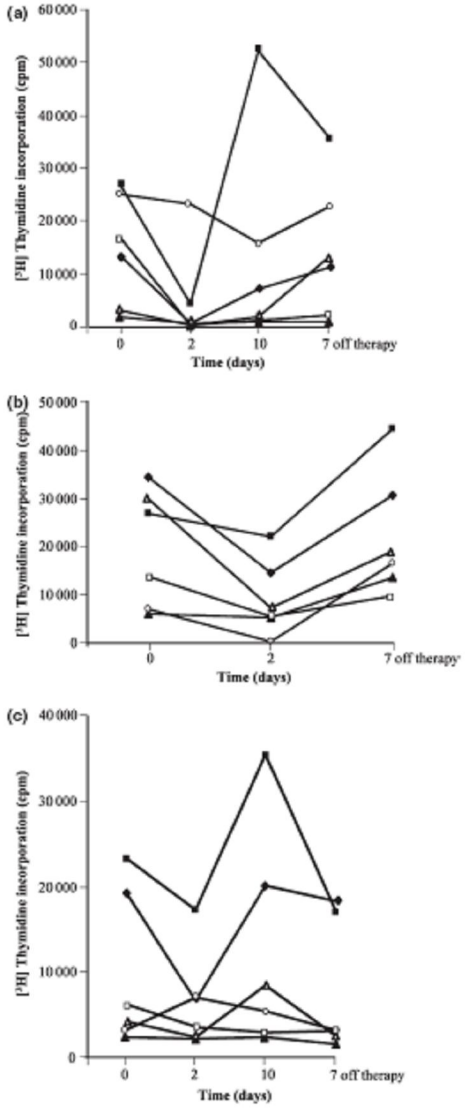

Figure 3 Proliferative response of peripheral blood mononuclear cells to mitogens in ataxia-teleangiectasia (A-T) patients. (a) Proliferative response to PHA. (b) Proliferative response to PMA plus ionomycin. (c) Proliferative response to PWM. Proliferation was evaluated before, during, and 7 days off therapy through [^{3}H]thymidine incorporation and expressed as cpm.

alterations in cell development during mid-gestation underlie the A-T-related ataxia [15,16]. Thus far, the worst aspect in the follow-up of A-T patients is the lack of an effective specific treatment for the neurological impairment.

In our study, the clinical evaluation was performed through the quantitative and well-validated SARA scale [13]. The highest clinical improvement was on speech disturbance, stance, and finger chase items. Of note, it should be pointed out that besides these parameters, in almost all patients, we could document a clinically objective improvement in the quality of motor coordination, an item not evaluated in the SARA assessment.

Magnetic resonance imaging of our A-T patients revealed several degrees of cerebellar atrophy, especially in vermian regions, which correlated, at some extent, with the disease duration, as previously reported [17]. In our study, the clinical benefit of therapy was inversely correlated with the degree of cerebellar atrophy, thus indirectly confirming that the loss of Purkinje cells correlates with the severity of the phenotype. However, the observation that two further patients, despite moderate grade atrophy, exhibited a clear amelioration of the neurological features provides good evidence that abnormalities at the molecular level precede gross morphological changes. Thus, we could argue that, during the initial phase of cell loss, biochemical and functional cerebellar abnormalities may still be modified, by interfering in the biochemical pathways.

As for the intimate molecular mechanism by which betamethasone led to this effect, thus far it is not possible to give a definitive interpretation. Neither the normal function of ATM in the nervous system nor the biological basis of the degeneration in A-T is known [15]. Although ATM seems to be neuroprotective in the tissue undergoing oxidative stress and apoptosis, the intimate molecular mechanism of its property is still uncertain [18]. As the effect of betamethasone was transient in nature, it should be argued that the intimate mechanism of this pharmacological action regards some still unidentified steroid-dependent biochemical event. This is not surprising, because a permanent reversion of the molecular alteration is not expected, and the drug presumably acts on the damage derived from ATM dysfunction. ATM also plays a role in the signal transduction network that modulates cell cycle checkpoints, genetic recombination, apoptosis, and other cellular responses to DNA damage [5,19,20]. Recently, increasing attention is being paid to the role of oxidative stress in the pathophysiology of A-T and other inherited ataxias [5]. In fact, whilst tumorigenesis in A-T seems to be linked to genetic instability, oxidative stress seems to play a major role in neuropathology [15,21,22]. The cerebellum has been proven to be a target for oxygen damage resulting in neurodegeneration. In ATM−/− mice, enhanced oxidative stress reduces life span and cells are more sensitive to radiations, thus suggesting that free radicals are important cofactors in the expression of a few A-T clinical features [23]. In keeping with this observation, administration of an antioxidant clearly prevented Purkinje cell death in *Atm*-deficient mice [9,18]. Taken together, these data provide evidence that oxidative stress contributes to the clinical phenotype of the disease [24]. It is, therefore, conceivable to hypothesize that the observation herein reported might be linked to a biochemical alteration of such a balance.

As for the immunological alterations observed during the betamethasone treatment, the increase of lymphocyte count is not surprising, in that it may be presumably linked to a direct effect of the drug on the bone marrow, favouring the dismission of mature cells into the peripheral blood. Again, the decrease in the proliferative response to mitogens of peripheral blood mononuclear cells may be explained on the basis of the immunosuppressive effect of steroids [25].

In conclusion, our observation confirms what recently reported on a single patient, and clearly indicates that for many years from the disease onset, neurological symptoms in A-T patients are susceptible of beneficial pharmacological intervention. However, due to the number of well-known adverse effects of corticosteroids, this therapy should be undertaken only after a careful consideration of the individual case. Most importantly, the rescue of the neurological function, at some extent, suggests that cell loss is an ultimate feature in A-T and that biochemical alterations, presumably reversible, precedes for a long time cerebellar atrophy. Further studies on the implicated mechanisms may open an important window on novel therapeutic agents capable of controlling neurological symptoms in A-T.

Acknowledgements

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Conclusive remarks

The clinical phenotype of A-T is quite heterogeneous with a surprising, and still unexplained, different impact of the pathogenic mechanism on the severity of immunological and neurological signs. Differently from lymphocytes, whose turn-over is continuous and characterized by the release into the peripheral stream of fully differentiated mature cells from bone marrow, the placement of Purkinje neurons occurs relatively early in human development (1). This would suggest that early alterations in cell development during mid-gestation underlie the A-T-related ataxia (12-13). Thus far, the worst aspect in the follow-up of A-T patients is the lack of an effective specific treatment for the neurological impairment. This observation confirms what recently reported on a single patient, and clearly indicates that for many years from the disease onset, neurological symptoms in A-T patients are susceptible of beneficial pharmacological intervention. However, due to the number of well-known adverse effects of corticosteroids, this therapy should be undertaken only after a careful consideration of the individual case. Most importantly, the rescue of the neurological function, at some extent, suggests that cell loss is an ultimate feature in A-T and the biochemical alterations, presumably reversible, proceed for a long time cerebellar atrophy. Further studies on the implicated mechanisms may open an important window on novel therapeutic agents capable of controlling neurological symptoms in A-T.

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

As above mentioned, neurological symptoms in A-T patients are susceptible of beneficial pharmacological intervention obtained trough betamethasone therapy. Conversely, the intimate molecular mechanism by which betamethasone led to this effect remains unclear, thus it is not possible to give a definitive explanation of this finding. However, the normal function of ATM in the nervous system nor the biological basis of the degeneration in A-T is known (12). Although ATM seems to be neuroprotective in the tissue undergoing oxidative stress and apoptosis, the intimate molecular mechanism of its property is still uncertain (14). As the effect of betamethasone was transient in nature, it should be argued that the intimate mechanism of this pharmacological action regards some still unidentified steroid-dependent biochemical event. This is not surprising, because a permanent reversion of the molecular alteration is not expected, and the drug presumably acts on the damage derived from ATM dysfunction.

ATM also plays a role in the signal transduction network that modulates cell cycle checkpoints, genetic recombination, apoptosis, and other cellular responses to DNA damage (6, 15-16). Recently, increasing attention is being paid to the role of oxidative stress in the pathophysiology of A-T and other inherited ataxias (6). In fact, while tumorigenesis in A-T seems to be linked to genetic instability, oxidative stress seems to play a major role in neuropathology (12, 17-18). The cerebellum has been proven to be a target for oxygen damage resulting in neurodegeneration. In *Atm-/-* mice, enhanced

oxidative stress reduces life span and cells are more sensitive to radiations, thus suggesting that free radicals are important cofactors in the expression of a few A-T clinical features (19). In keeping with this observation, administration of an antioxidant clearly prevented Purkinje cell death in *Atm*-deficient mice (9, 14). Taken together, these data provide evidence that oxidative stress contributes to the clinical phenotype of the disease (20). It is, therefore, conceivable to hypothesize that the reported observation might be linked to a biochemical alteration of such a balance.

With this regard, in the second phase of this study we evaluate whether the beneficial effect of betamethasone therapy could be mediated by interference in reactive oxygen species (ROS) generation/neutralization process. Thus, we measured in this small cohort of A-T patients direct markers of ROS production, during a short-term steroid treatment.

Of note, ATM protein, after its autophosphorylation, activates multiple substrates involved in cell cycle regulation, such as p53 and, eventually, leads, cooperating with other DNA repairing factors, to cell cycle arrest. ATM is also involved in sensing and modulating intracellular redox status, even though it is not clear whether ATM itself is directly involved in ROS production (16, 21). However, ATM deficiency causes oxidative damage to proteins and lipids in brain, testes and thymus. Furthermore, *Atm*-deficient mice exhibit genomic instability and hypersensitivity to ionizing radiation and other treatments that generate ROS (22).

In keeping with this, in *Atm*-deficient mice the overexpression of superoxide dismutase 1 (SOD), an enzyme involved in hydrogen peroxide production, exacerbated certain features of the A-T phenotype (23). These observations point to additional roles for ATM in cellular metabolism other

than its direct role in the response to DSBs induction, such as its involvement in ROS balance.

Cells have developed a wide array of protective mechanisms against ROS, including small reducing molecules, antioxidative enzymes and damage/repair systems (24). One of the small reducing molecules is glutathione (GS), which appears in the cells in both its oxidized and reduced (GSH) forms. It works in detoxifying specific ROS by itself or in combination with other enzymes, such as SOD, which converts superoxide radicals into H_2O_2 and O_2, catalase, glutathione peroxidase (PHGPx) and glutathione-S-transferases. Oxidants overproduction and/or dysfunction of endogenous antioxidant defenses result in oxidative stress induced injury with damage to all the major classes of biological macromolecules, such as nucleic acids, proteins, lipids and carbohydrates. The CNS is particularly vulnerable to oxidative stress due to its high rate of metabolism and to the disproportionately low levels of its oxidative defense mechanisms (25). The increased and unopposed ROS production can lead to neurotoxicity resulting in neural damage and eventually in cell death.

Within the observation of the neurological amelioration during steroid treatment in A-T patients, we found that this improvement was inversely correlated with the age and severity of cerebellar atrophy and that the highest basal GSH values were present in PBMC from those patients who better responded to the betamethasone therapy. Furthermore, it is interesting to note that the behaviour of ROS levels in the patient, who better responded to the treatment, correlated with the clinical improvement. In particular, ROS levels decreased during the phase of the improvement and returned to normal seven days off the therapy, paralleling the clinical worsening. During this study, we

analyzed also lipid peroxidation and found that this phenomenon occurred only in the patient who did not exhibit any clinical improvement during the therapy and, importantly, that it was associated with the most severe cerebellar atrophy. These findings suggested that the antioxidative protective GSH system plays a role in the protection from the cerebellar atrophy and may be implicated in the improvement of cerebellar functions during short-term betamethasone therapy.

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

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Ataxia-teleangiectasia, betamethasone, neurological features, reactive oxygen species

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

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

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

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

Introduction

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

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

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

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

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

Subjects and methods

Patients

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

Cell isolation and treatment

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

Determination of lipid peroxidation

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

Evaluation of intracellular glutathione

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

Measurement of intracellular ROS

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

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

Results

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

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

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

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

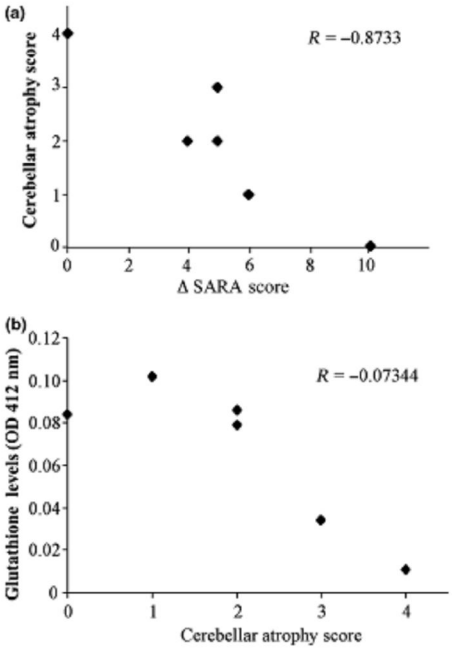

Figure 1 Correlation between neurological amelioration during steroid therapy, cerebellar atrophy and glutathione levels. (a) Indirect correlation between cerebellar atrophy score and the improvement of cerebellar functions, expressed as Δ SARA score, in ataxia-telangiectasia patients. (b) Indirect link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

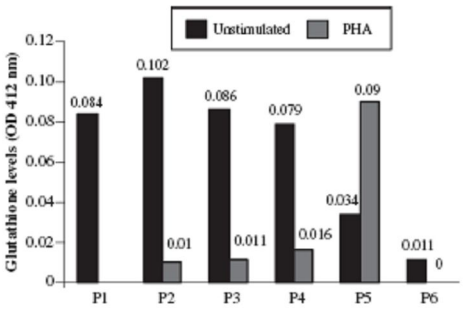

Figure 2 Cellular levels of glutathione in peripheral blood mononuclear cells, unstimulated or stimulated with PHA, obtained from ataxia-telangiectasia patients. In five of six patients PHA stimulation induces a significant decrease of glutathione levels.

The effect of ROS hyper-production on lipid peroxidation

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

Evaluation of intracellular ROS levels in lymphocytes

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

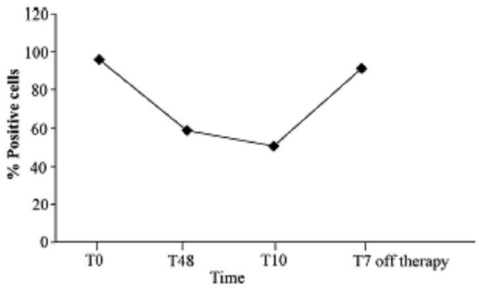

Figure 3 Intracellular reactive oxygen species (ROS) levels in peripheral blood mononuclear cells derived from the better clinical responder (P1) to betamethasone treatment. A substantial decrease in ROS levels is observed during steroid therapy.

Discussion

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

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

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

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

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

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

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

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Conclusive remarks

Even though A-T is non-curable we had previously demonstrated an improvement of the cerebellar functions in 6 A-T patients after a short-term betamethasone therapy (0.1 mg/kg/day for 10 days) (26). Although the clinical response to betamethasone was evident, there was no evidence indicating the intimate mechanism of action of this drug in A-T. In this second study, on the basis of our finding, we concluded that betamethasone response in A-T patients is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. The importance of this study relies principally on the severity of the neurological signs. In fact, this childhood disease is characterized by progressive impairment of gait and speech, oculomotor apraxia (inability to move the eyes from one object to another), oculocutaneous telangiectasia (dilated blood vessels), cerebellar atrophy, sterility and radiosensitivity (12). Approximately, 10%–15% of A-T patients develop cancer, most frequently acute lymphocytic leukemia and lymphoma. The prognosis for survival is poor, and individuals with this disease usually die in their teens. The earliest clinical manifestation of A-T is ataxia (2). By approximately 4 years, deterioration of gross and fine motor skills occurs; eye movements are characterized by a series of small jumps rather than in a single smooth motion (oculomotor apraxia); choreiform movements of the hands and feet are observed, difficulty with chewing and swallowing occur and drooling is common. A-T is primarily a syndrome of progressive cerebellar ataxia but more diffuse changes to the CNS are also evident. Cerebellar degeneration in A-T manifests as dystrophic changes involving the dendrites and axons of Purkinje cells and ectopic Purkinje cells are evident. Of all the features of A-

T the progressive cerebellar neurodegeneration is the most debilitating. By the end of the first decade ataxia has progressed to the extent that the child is confined to a wheelchair and may have poor control of head and torso leading eventually to spinal muscular atrophy. Thus, any effective treatment for A-T would ideally involve prevention or at least slowing of the progressive neurodegeneration. As mentioned above, at present there is no therapy available to cure or prevent the progress of A-T, but it is possible to alleviate some of the symptoms associated with immunodeficiency and deficient lung function but neither the cancer predisposition nor the progressive neurodegeneration can be prevented. In conclusion, this multisystem disease requires intervention to: (i) halt progressive neurodegenerative changes; (ii) reduce the risk or treatment of tumours; (iii) correct immunodeficiency; (iv) alleviate bronchial complications. There is no cure for the progressive neurodegeneration with conventional therapies but some promise exists. Most importantly, the rescue of the neurological function, at some extent, suggests that cell loss is an ultimate feature in A-T and those biochemical alterations, presumably reversible, proceed for a long time cerebellar atrophy.

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

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CHAPTER 4

The T-cell ontogeny: from positive to negative selection

A normal T-cell ontogeny and the generation of a heterogeneous peripheral T-cell pool are essential for a normal immune response. To date, the "primary" organ for the differentiation and selection of T cells is represented by the thymus that, providing a complexity of specialized cells as epithelial and dendritic cells, macrophages and fibroblasts of the stroma (1), influences each stage of T-cell precursor maturation program (2).

One of the important regulators of thymic epithelium, whose integrity is crucial for T-cell maturation, is represented by the product of the nude gene locus, FOXN1, above extensively described.

During the maturational process, first HPCs emerge from bone marrow and reach the thymus where a complex series of maturational proceedings take place, an event known as positive selection (3).

Within the thymus, recently formed T cells also undergo negative selection, or central tolerance, whose hallmark is the clonal deletion of self-reactive T clones, being this process efficiently mediated by bone-marrow derived dendritic cells (DCs). In the thymus, the negative selections distinguishes T cells with low or high affinity for self-antigens, whose transcription is induced by the autoimmune regulator (AIRE) gene product, and self-MHC and eliminate those clones with more affinity for self. Really, some autoreactive clones could escape thymic control; these cells are however controlled in the periphery. The establishment of the peripheral tolerance is attributable to the induction of functional anergy, deletion by apoptosis, and the suppressive actions of regulatory T

lymphocytes (Treg). Each of these pathways is essential for maintaining self-tolerance.

Mutations in the *AIRE* gene lead to defective clonal deletion and to the multi-organ syndrome known as autoimmune – polyendocrinopathy – candidiasis – ectodermal distrophy syndrome (APECED).

§ 4.1 FOXN1 mutation abrogates pre-natal T-cell development in humans

The genetic study of human SCID has unravelled in the last 2 decades important issues concerning the rules that govern lymphocyte development and function (4). 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 (5). However, although an extensive knowledge exists about thymus role in T-cell development, a fully comprehension of this mechanism remains unexplained. In particular, important issues remain unclear about the involvement of different non-lymphoid tissues such as skin, gut, or liver in T-cell ontogeny. A second thymus-like organ able to support T-cell full maturation and to reconstitute T-cell compartment in athymic nude mice was found (6). In humans, the DiGeorge syndrome has been long considered the prototype of an athymic disorder, even though in these patients some mature type T cells are present making waver the dogma of the thymus as the unique fundamental organ in T-cell ontogeny. In mice and rats, spontaneous mutations FOXN1 transcription factor gene, mapping on chromosome 17, revealed that Foxn1 is essential for thymic and skin epithelial differentiation and hence thymopoiesis (7-9). In 1966, this athymic animal model was referred as the Nude/SCID phenotype due to the presence of congenital alopecia (10-11). However, this spontaneous animal disease profoundly differs in the immunological aspects from the human DiGeorge syndrome. In 1996, the human equivalent of the mouse Nude/SCID has been described in a small

geographically isolated community in south Italy in 2 sisters with the R255X homozygous mutation in *FOXN1* gene (12-13). In this community, the identification of a number of heterozygotes for the same mutation suggested the need to offer to the population a pre-natal diagnosis program for the disease.

During the genetic counselling, a fetus at risk has been identified. *FOXN1* gene sequence analysis revealed the homozygous C-to-T transition at nucleotide position 792, leading to the nonsense R255X mutation. Protein expression analysis by immunohistochemistry on fetal skin fragments revealed the absence of FOXN1 protein as expected on the basis of the molecular alteration. Since in certain circumstances, such as immunodeficiencies (14), maternal cells may cross the placenta and engraft into the fetus in utero, all samples were analyzed after exclusion of maternal cell contamination.

The study of the immune system in this Nude/SCID fetus gave the unique opportunity to gain further insights into the development of T lymphocytes in humans in the absence of the thymus and in the absence of the FOXN1 transcription factor.

Because T cells originate from common multipotent HPC, to exclude a direct involvement of HPC the presence of $CD34^+$ cells at biochemical level was evaluated. $CD34^+$ cells were comparable to the control fetus, 1% of $CD45^+$ hematopoietic cells and, consistently with this, no abnormalities in the development of B (65%) and NK (25%) cells were found.

The concomitant assembly of CD4 and CD8 molecules on thymocyte surface or their putative individual expression in conjunction with CD3 are

markers of discrete stages of T-cell development. The evaluation of nude cord blood cells revealed that the lack of the thymus led to a complete absence of $CD4^+$ cells (0.3%) compared to 72% of the control fetus. Differently, $CD8^+$ lymphocytes were present (10.3%), although among this population cells co-expressing CD3 were considerably low (2.6%), whereas $CD3^+CD8^+$ in the control were 13.7%. Of note, approximately 40% of these cells displayed a naive phenotype as assessed by the expression of CD45RA isoform.

T cells are also distinguished by their cell surface TCRs. A substantial reduction of T cells bearing TCRab (1.6% vs 93% of the control) was observed. Differently, 6.5% of cells expressed TCRgd, but the majority of these cells were $CD3^-$ cells. Moreover, 74% of TCRγδ bearing cells expressed CD8ab heterodimer. Again, the diversity of the repertoire was ensured at some extent by the few $CD8^+$ cells. The evaluation of Vb usage among $CD4^+$ and $CD4^-$ cells revealed that the generation of TCR diversity was consistently impaired. In the $CD4^+$ and $CD4^-$ subsets only 3 and 1 families, respectively, had a gaussian profile. In the $CD4^-$ subset 4 families predominated, accounting for more than 10% of the total. In this subset, the bV25 was monoclonal and represented 10% of the entire repertoire. As expected, $FOXN1^{-/-}$ cord blood mononuclear cells showed absent proliferative response after stimulation with anti-CD3 or phytohemagglutinin (PHA).

To define the extrathymic site of lymphopoiesis of these few $CD8^+$ cells, gut mucosa was studied, as the best candidate of primordial lymphopoiesis. In the intestine $CD3^+$ and $CD8^+$ cells but no $CD4^+$ lymphocytes in the human athymic fetus were found. As shown in the

figure, $CD3^{+}$ cells in the gut of $FOXN1^{-/-}$ fetus were normally distributed across the gut mucosa, even though not uniformly as expected. This finding argues in favour of a non thymic origin of intestinal lymphocytes.

This study is ready to be submitted to *Nature*, due to the importance of the finding to study the immune system in humans in the absence of a functional thymus.

Conclusive Remarks

This study provide a direct evidence of the crucial role of FOXN1 in 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 gd T-cell production. Since FOXN1 is only expressed in the thymus and skin, our data strongly indicate that skin epithelial cells play a necessary role in T-cell ontogeny, thus explaining the reason of the presence of a considerable number of mature T cells in DiGeorge syndrome. These findings also argue in favour of gut mucosa as an extrathymic site of $CD8^+$ cell development, thus confirming former observations (15-16). Remarkably, the development of this subset occurs in a thymus independent fashion.

§ 4.2 High intrafamilial variability in APECED syndrome: a peripheral tolerance study

The genetic cause of many rare autoimmune diseases have been already identified and most of these disorders are a result of an intricate relationship between environmental and genetic factors, resultant in a deregulation of central and peripheral tolerance, that led to auto reactive pathogenetic T and B clones.

The intriguing evidence that the genetic background predisposes to the autoimmunity, but it doesn't define the specific target of the disease, demonstrate that there are several factors that influence the phenotypical characteristics (17-20).

Autoimmune Polyendocrinopathy – Candidiasis – Ectodermal - Distrophy (APECED; MIM 240300), or autoimmune polyglandular syndrome type I, is a rare autosomal recessive disease characterized by a set of three abnormal features: chronic mucocoutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency. However, most patients also routinely exhibit a variable number of other autoimmune diseases. APECED is caused by mutations of a single gene, named autoimmune regulator (AIRE), which maps to 21q22.3 (21) and encodes a 55-kDa protein that acts as a transcription factor (22). Animal models of APECED have revealed that AIRE plays an important role in T cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. Thus, the absence of AIRE results in impaired clonal deletion of self-reactive thymocytes (23). Over

60 mutations have by now been localized in the AIRE genes of different APECED patients but the different mutations have not to date been convincingly associated with particular disease manifestations (23).

Although the disease is monogenic in transmission, APECED patients show a variable range of pathological manifestations, with each patient presenting a different constellation of affected organs and auto-antibodies specificities. However, certain targets of the autoimmune attack are near constant, such as the adrenal and parathyroid glands, while other manifestations are less frequently observed (e.g., thyroiditis or T1D, which are present in 2–12% of cases) (24). Recent analysis have revealed that an effect of additional genetic loci, in particular the human leukocyte antigen (HLA) complex, is restricted only to few disease manifestations (25-26). Moreover, recent evidences indicate that also a defect in the regulatory T cells compartment (Treg) could be involved in the pathogenesis of APECED (27). So far, the reasons of the high phenotypical variability of APECED remain still unclear.

This study was aimed to evaluate whether genetic, immunological and environmental factors may be involved in the modulation of the disease in two siblings, born from consanguineous parents, with identical genotype and extremely different phenotype by studying the exposure to infectivological triggers, the HLA aplotype and several mechanisms of peripheral tolerance.

Subjects

Case 1

The boy first presented at the age of 5 years with severe asthenia, alopecia, and an urticaria-like erythema during a fever. Physical examination

revealed the presence of vitiligo, alopecia, nail dystrophy, oral candidiasis hypertransaminasemia and hepatosplenomegaly. Autoimmune hepatitis (AIH) was scored as probable (score 12) on the basis of the recommendation of the international AIH group.2 Laboratory investigations showed hypocalcaemia (1.3 mmol/l) and hyperphosphatemia (2.3 mmol/l) with undetectable levels of PTH, leading to the diagnosis of hypoparathyroidism. He was started on calcium and calcitriol therapy. Over the next three months the patient developed autoimmune thyroiditis with severe hypothyroidism (TSH 251 mIU/L, Free-T4 6.3 pmol/l) and he started L-Thyroxine therapy. An increase in plasmatic renin levels (216 pg/ml, normal range 1.8-33 pg/ml) in the presence of adrenal auto-antibodies, confirmed the diagnosis of adrenal insufficiency. He also presented gastrointestinal symptoms with abdominal distension accompanied by alternating diarrhea and constipation and presence of auto-antibodies against TPH and AADC, commonly associated with gastrointestinal alterations in APECED patients. Atrophic gastritis was diagnosed on the basis of persistently elevated levels of gastrinemia (765 pg/ml, normal range 0-80 pg/ml), presence of parietal cells Abs (PCA) and histological findings of gastric body mucosa atrophy.

Six months after the onset of this accelerated phase, the patient suddenly developed a severe neurological symptomatology with neuroradiological findings suggestive of Posterior Reversible Encephalopathy Syndrome (PRES), a life-threatening event never described before in APECED patients.

Case 2

The girl, the younger sister of case 1, presented at the age of 4 years with hypoparathyroidism, diagnosed on the basis of hypocalcaemia, hyperphosphatemia and undetectable levels of PTH, and chronic mucocutaneous candidasis. No other signs or symptoms of APECED were revealed at physical examination or biochemical investigations. During her follow-up, through the next two years, she did not develop any other features of the disease.

Direct sequencing of the patients' AIRE gene revealed in both the siblings the presence of the same complex homozygous mutation in intron 1 consisting of a substitution of IVS1 + 1G by C accompanied by a single nucleotide deletion at IVS1 + 5G residue (IVS1 + 1G>C; IVS1 + 5delG). The parents were both heterozygous for the same mutation and had no features of autoimmunity. This mutation represents an uncommon mutation of AIRE and, so far, has been described in heterozygous state with the R257X in a single individual from Poland.

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As previuosly described, circulating auto-antibodies paralleled in each patient the clinical phenotype, and strongly supporting the autoimmune mechanism at the basis of the pathogenesis of APECED. Moreover, the

evaluation of specific response to infectivological triggers, showed no substantial differences that could influence the severity of the disease in that the younger sister, which presented a milder phenotype, was more exposed to viral infections.

To define the possible contribution of distinct peripheral tolerance mechanisms in the high variability observe in the two patients, we also analyzed the resistance to Fas-induced apoptosis and the number of CD4+CD25+ cells indicative of the presence of Treg population. In particular, cell death assay after Fas stimulation revealed that the resistance to Fas-induced apoptosis was comparable between the siblings and normal as in the controls. Flow cytometric assays revealed a comparable number of CD4+CD25+ T cells in the two siblings, but in both cases reduced as compared to controls.

Being NK cells involved in the autoimmunity attributable to failure in deletion by cytolitic protein such as Perforin (PRF1), we analyzed NK cells activity that resulted comparable to controls in both the cases. Moreover, no mutations in the coding region of PRF1 gene were identified in both the siblings. It was notable that the molecular study of the PRF1 gene disclosed a heterozygous nucleotide substitution in PRF1 exon 3 resulted in the Ala273Ala silent mutation. This substitution was only found in the Case 1.

Conclusive Remarks

Here we reported the case of two siblings affected with APECED that showed a high intra familiar variability of phenotype despite the same genotype of the AIRE gene. Although APECED is generally considered a monogenic disorder, it is characterized by a wide variability of expression with each patient presenting a different constellation of affected organs and autoAb specificities.

As other autoimmunity disease, APECED is the result of alterations at different levels, as demonstrated by the remarkable north/south gradient in the susceptibility to autoimmune diseases that suggests that environmental factors could be involved (28).

Studies of Aire knockout mice have provided direct evidence that Aire has a vital role in preventing autoimmunity. In particular, APECED animal models revealed that Aire plays an important role in T-cell tolerance induction in the thymus, mainly by promoting ectopic expression of a large repertoire of transcripts encoding proteins normally restricted to differentiated organs residing in the periphery. Thus, the absence of AIRE results in impaired clonal deletion of self-reactive thymocytes, which escape into the periphery and attack a variety of organs (23).

The extreme variability of APECED presentation, even between siblings with the same genotype, leads to speculate that other mechanisms, beyond AIRE itself, could be involved in the clinical expression of the disease.

We observed two siblings patients with the same genetic AIRE mutations, but with a spectrum of target organs completely different.

Although it is well known that APECED is characterized by extreme variability of phenotype, the reasons of this phenomenon remain still unclear.

In particular, being the infectious agents represent a potent stimulus for the immune system and may contribute to select auto reactive T cells in susceptible subjects, through molecular mimicry (29-31), bystander activation (32-33) and epitope spreading (34), we examined the associated infections in both patients, but they show an identical infective story. Moreover, we analyzed NK activity and eventual genetic alterations in PRF1 gene. The results of this analysis showed a comparable NK activity any alterations in PRF1 gene for both patients. It is likely that a not-screened mutation in the intronic or in the promoter region of PRF1 gene could influence the different phenotype observed in the siblings.

Furthermore, on the other side of the central tolerance there are other mechanisms that maintain tolerance to self, such as the induction of functional anergy, deletion by apoptosis, and the suppressive actions of Treg. On the bases of these mechanisms we analyzed in particular the resistance to apoptosis by cell death assay with Fas, the number and the functionality of Treg, attributable to a normal expression of the transcription factor Foxp3. During this analysis we didn't note any variations between the two sibling patients.

In summary, we found a high intrafamiliar variability in two siblings affected with the same mutation of AIRE gene. The results imply that the analyzed mechanisms did not influence the phenotypic spectrum of APECED.

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Technologies

Reagents

Recombinant human GH (rGH) was obtained from Serono (Saizer 4). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences. The Abs anti-gc, anti-JAK3, anti-b-actin, anti-phosphotyrosine ERK, anti-histone 3 (H3), anti-phosphotyrosine, anti-STAT5 were purchased from Santa Cruz Biotechnology. Acrylamide and bisacrylamide were obtained from Invitrogen. Prestained molecular mass standards were obtained from Bio-Rad. The small interfering RNA (siRNA) duplexes specific for gc 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. The Ab anti-JAK2 was purchased from Cell Signaling Technology. The neutralizing IgG1 anti-gc mAb was purchased from R&D Systems. An IgG1 isotype-matched anti-CD3 mAb (Leu 3, UCHT1 clone) was purchased from BD Biosciences. Epidermal growth factor was purchased from BD Biosciences and used at the concentration of 100 ng/ml. Except where noted, other reagents were from Sigma-Aldrich.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from four X-SCID patients and normal donors and heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by EBV immortalization

of patients and control PBMC using standard procedures. In all cases, gc mutations led to the absence of protein expression. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM/L L-glutamine (Invitrogen Life Technologies), and 50 microg/ml gentamicin (Invitrogen Life Technologies), and cultured at 37°C, 5% CO2. In BCL transduction experiments, the pGC2Rg retroviral vector was used to transduce X-SCID BCLs with wild-type gc as previously described. Transduced cells were selected in the neomycinanalog G418 (Cellgro). NIH 3T3 fibroblasts were used in a few experiments.

siRNA transfection

Preparation of the cells before Lipofectamine 2000 transfection was performed according to the manufacturer's recommendations. Briefly, for each transfection 1 X 10^6 BCLs in 1 ml were treated with 20 microl of 50 microM of siRNAs specific for the gc or equal amount of the control nontargeting siRNA. The siRNAs were solubilised 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.

Proliferative assay

BCLs (1x105 cell/200 microl well) were cultured triplicate in 96 well Ubottom microtiter plates (Falcon; BD Biosciences) with or without rGH at reported concentrations for 4 days. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 microCi of [3H]thymidine (Amersham Biosciences) 8 h before harvesting. In neutralization experiments, control EBV cells were preincubated with the neutralizing mAb 284 at the concentration of 6 ng/ml for 3 h or with the IgG1 isotype-matched Ab (Leu 3). Where indicated for proliferative assay were used common mitogens.

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.

CFSE labelling

Cell proliferation was measured by the cell surface stain carboxyfluorescein diacetate succinimidyl ester (CFSE). BCLs (1 X 10^6) were labelled with 1.7 μM CFSE in PBS just before culturing for the indicated times. After 2 min at room temperature, BCLs were washed in FBS and phosphate-buffered saline (PBS) and cell division accompanied by CFSE dilution was analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson).

Flow cytometry

The expression of GHR was detected using specific rabbit Abs (Santa Cruz Biotechnology) by indirect immunofluorescence using a second-step

incubation with FITC-conjugated donkey anti-rabbit Abs (Pierce). After washing in PBS, cells were incubated for 20 min with the specific Abs and 30 min with secondary Abs. After staining, all samples were washed in PBS and acquired on the FACScan flow cytometer (BD Biosciences) using Lysis I software.

Cell stimulation and protein extraction

Before hormone treatment, the cells were made quiescent through incubation in RPMI 1640 minus serum for 8–12 h. GH was used at 37°C at a concentration of 500 ng/ml in RPMI 1640 for the reported time. Incubations were terminated by washing cells with ice-cold PBS (BioWhittaker) followed by solubilization in 100 microl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadatum (Na3VO4), 5 microg/ml leupeptin, and 5 microg/ml aprotinin. The cell lysates were stored at -80°C for Western blot analysis. Nuclear extracts were prepared by previous described method and were subsequently mixed with sample buffer.

Western blot and immuneprecipitation

Immunoblotting using phosphotyrosine mAb was performed as previously reported. Immunoblotting using specific Ab was performed according to the vendor protocols. In brief, protein samples separated by SDS-PAGE were transferred onto Mixed Cellulose Esters membranes (Immobilon-NC Mixed Cellulose Esters 0.45 _m; Millipore). The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 10% BSA in

wash buffer (10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20). 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.

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.

Genetic counseling

A genetic counseling program was offered to the village population from which the 2 probands affected with the Nude/SCID phenotype were 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. The genetic counseling also included taking a detailed family history and drawing a family tree.

Mutational Analysis

After written informed consent was obtained from parents and upon approval of Institutional Ethical Committee, genomic DNA was extracted following standard procedures from amniocytes and processed anonymously.(Miller, 1988 #1130) This procedure was performed in a laboratory with "CLIA certification" and *FOXN1* DNA analysis was performed according to a polymerase chain reaction assay previously described. (Adriani, 2004 #1235) Briefly, a PCR fragment containing exon 5 of the *FOXN1* gene was amplified using the primers: Exon 5F: 5'-CTTCTGGAGCGCAGGTTGTC-3' and Exon 5R: 5'-TAAATGAAGCTCCCTCTGGC-3'. Aliquot of the samples to be studied were digested with the restriction enzyme Bsr I and the product was

electrophoresed on 1.5% agarose gel. PCR fragments positive to the digestion with Bsr I were then purified on Edge Centriflex columns (Edge BioSystems) and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer from Applied Biosystems Inc. (Perkin Elmer). After informed consent fetus underwent physical examination by our pathologist in order to define whether *FOXN1* homozygous alteration was associated with defects in the differentiation process and fetus development.

For *AIRE* mutation analysis genomic DNA was isolated from peripheral blood lymphocytes of both patients. Primers were designed to amplify each of the 14 exons of the AIRE gene and its intronic flanking sequences (GenBank accession no. AB006684). PCR conditions were optimized using various magnesium concentrations and annealing temperatures. 40 ng of genomic DNA was run in each PCR reaction. PCR products were then purified from agarose gel by a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany), automatically sequenced using Big Dye terminator (Applied Biosystems, Foster City, CA), and then run on an ABI 3100 machine (Applied Biosystems).

A-T Patients

Blood samples were obtained from six consecutive patients (3 males), of 5, 6, 14, 15, 27 and 29 years of age, respectively (mean age 16.3 years, range 5-29 years), affected with A-T. The patients, upon written consent, received a cycle of oral betamethasone at the dosage of 0.1 mg/kg/day for 10 days divided every 12 hours. No antioxidants, as N-acetyl cysteine, vitamin E or alphalipoic acid, were given to the patients during the two

years preceding the trial. The clinical evaluations were carried out before therapy (T0), 48 h from the beginning (T48), at the end of the therapy (T10d) and, eventually, 7 days from the withdrawal (T7 off therapy). The neurological evaluation was performed through the Scale for the Assessment and Rating of Ataxia (SARA) (see appendix E1 on the neurology Web site at http://www.neurology.org/cgi/cintent/full/66/11/1717/DC1). In particular, in this study we utilized the variation in SARA Score (D), considering that the higher D is the higher amelioration. Cerebellar atrophy score was calculated as follows: 0 score= absence of cerebellar atrophy; 1 score= absence of cerebellar atrophy with moderate pontocerebellar angle cisterns enlargement; 2 score= moderate atrophy involving mostly both superior and inferior portion of the vermis and, at a lesser extent, the cerebellar hemispheres with moderate enlargement of periquoral spaces; 3 score= severe atrophy of superior portion of vermis and moderate atrophy of inferior part of vermis; severe atrophy of superior and lateral portion of cerebellar hemispheres and moderate atrophy of inferior hemispheres; 4 score= global and severe atrophy of the superior and inferior part of vermis and the whole cerebellar hemispheres with marked fourth ventricle enlargement.

Cell isolation and treatment in redox studies

Mononuclear cells (PBMC) were obtained from A-T patients and normal donors heparinized peripheral blood by Ficoll-Hypaque (Cambrex, Milan, Italy) density gradient centrifugation. Cells were maintained in RPMI 1640 (Cambrex, Milan, Italy) supplemented with 10% FBS (Cambrex,

Milan, Italy), 2mM/L L-glutamine (Invitrogen Life Technologies, Milan, Italy), and 50 μg/ml gentamicin (Invitrogen Life Technologies, Milan, Italy), and cultured at 37°C, 5% CO_2 without stimuli or stimulated for 48 h with PHA (8mg/ml) and then collected.

Determination of lipid peroxidation

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

Evaluation of intracellular glutathione

The cells were lysed by freeze and thaw. Cellular levels of GSH were determined using DIGT-250 GSH colorimetric assay kit (BioAssay Systems, California, USA). Cells ($0.8x10^5$) were treated for 48 h with various agents and collected by centrifugation at 1300 g for 10 min at 4°C. All the samples were analyzed following manufacturer's instructions. Optical density (OD)-values were read at 412 nm. The samples were normalized for cell number.

Measurement of intracellular ROS

The cellular ROS accumulation was measured using the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) method. DCFH-DA is a non-fluorescent compound, and it can be enzymatically converted to highly

fluorescent compound, DCF, in the presence of ROS. After and before exposure to betamethasone, $1x10^6$ cells were treated with 5 µM DCFH-DA at 37°C for 20 min, at 5% CO2, washed with PBS, collected and analyzed immediately on a FACScan flow cytometer (Becton Dickinson, New Jersey, USA) equipped with an argon laser emitting at 488 nm. Cell Quest software (Becton Dickinson, New Jersey, USA) was used to acquire and evaluate all the events.

Cell death assay

Fas-induced cell death was evaluated, as previously reported, on T-cell lines obtained by activating PBMCs with phytohemagglutinin (PHA) at days 0 (1 mg/mL) and 13 (0.2 mg/mL) and cultured in RPMI 1640 medium þ 10% fetal bovine serum (FBS) þ recombinant interleukin-2 (rIL-2) (2 U/mL) (Biogen). Fas function was assessed at day 19. Cells were incubated with control medium or anti-Fas MAb (1 mg/mL) (CH 11 clone, UBI) in the presence of rIL-2 (2.5 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by trypan blue exclusion test.

Summary

The field of the clinical description, genetic characterization, and immunological investigation of novel PIDs, born in the 1950s, in the last twenty years of the 20th century has revealed a formidable numbers of scientific discoveries. Many scientific papers have been published on the molecular and cellular basis of the immune response and on the mechanisms involved in the correct ontogeny of immune system components. Although today we know the genetic and molecular basis of those principal mechanisms involved in the immune response, this field retains a great potential for growth.

During the three years of my PhD program I have contributed in some measure to clarify some "*Novel aspects in Immunodeficiencies*", through the combination of clinical, cellular, functional and molecular approaches.

In particular, studying a group of atypical patients with X-SCID I contributed to demonstrate for the first time a previously unappreciated functional interaction between the GHR apparatus and the gc. Furthermore, this knowledge helped me to reveal a prior unidentified property of the common gc trasducing element. I could demonstrate, in fact, that this subunit is able to influence the cell-cycle progression, spontaneous or GH-induced, in a concentration dependent manner.

Moreover, I spent my PhD also to better characterize at both molecular and clinical levels the human Nude/SCID phenotype. In this context, I could suggest for the first time a functional role of FOXN1 transcription factor in the development and differentiation of the central nervous system.

In addition, among those PIDs whose causing genes are not selectively expressed in the hematopoietic compartment, I contributed to the study first of the effects of steroid treatment in patients affected with Ataxia telangiectasia, currently without an effective treatment, and after of the role of oxidative stress in its beneficial therapy.

During my PhD I also paid a particular attention to T-cell ontogeny defects affecting both the positive and negative selection processes. In this field I had a unique opportunity to study T-cell development in humans in the absence of a functional thymus; this study led to the identification of some subsets of T-cell able to mature in extrathymic sites. In the context of the negative selection process, I studied the role of peripheral tolerance mechanisms in the high phenotypic intra-familiar variability existing in the APECED syndrome.

Overall, all my studies were designed in order to clarify some unknown mechanisms underlying the functionality and the development of the immune system well as to study the basis of some regulatory networks governing the interactions existing between the immune and other systems.

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I would like to thank all the people who helped me in these three years of fellowship because without their support I couldn't end my PhD so well. In particular, I would like to thank Prof. Claudio Pignata for his support, his teachings and, especially, because he believed in my abilities and made me love this job. I wish to thank all my colleagues (Ilaria Russo, Teresa Broccoletti, Anna Fusco and Ilaria Vigliano) who supported and tolerated me all this time. Really, without them the last three years could not be so dynamic, productive, great and so full of friendship. Last but not least, I'd like to thank all my family, Antonio and all my friends because this PhD thesis is above all the product of their love.

Appendix I

Abbreviations

ADA	=	Adenosine Deaminase
APC	=	Antigen Presenting Cells
A-T	=	Ataxia Telangiectasia
ATM	=	Ataxia Telangiectasia Mutated
APECED	=	Autoimmune – Polyendocrinopathy – Candidiasis – Ectodermal Distrophy
AIRE	=	AutoImmune REgulator
BCL	=	B Cell Line
BMP	=	Bone Morphogenetic Proteins
CSP	=	Cavum Septum Pellucidum
CNS	=	Central Nervous System
CSF	=	CerebroSpinal Fluid
gc	=	Common Gamma Chain
DCs	=	Dendritic Cells
DSB	=	Double-Strand Break
DC	=	Dyskeratosis Congenita
FOX	=	Forkhead bOX
GS	=	Glutathione
PHGPx	=	Glutathione Peroxidase
GVHD	=	Graft-Versus-Host-Disease
GH	=	Growth Hormone
GHR	=	Growth Hormone Receptor
HPC	=	Hematopoietic Precursor Cell
IKK	=	IkB Kinase
IPEX	=	Immunodysregulation Polyendocrinopathy Enteropathy X-linked
Ig	=	Immunoglobulin
IL	=	InterLeukin
IVIG	=	IntraVenous ImmunoGlobulin
JAK	=	Janus Associated Kinase
KGF	=	Keratinocyte Growth Factor
MRI	=	Magnetic Resonance Imaging
NK	=	Natural Killer
PRF	=	Perforin
PI3K	=	Phosphatidyl Inositol 3 Kinase
PHA	=	Phytohemagglutinin
PID	=	Primary ImmunoDeficiency
PKB, Akt	=	Protein Kinase B
ROS	=	Reactive Oxygen Species
GSH	=	Reduced Glutathione

Treg	=	regulatory T lymphocytes
SARA	=	Scale for the Assessment and Rating of Ataxia
SGK	=	Serum/Glucocorticoid-Regulated Kinase
SCID	=	Severe Combined ImmunoDeficiency
siRNA	=	Short Interfering RNA
STAT	=	Signal Trasducer and Activator of Trascription
SMAD	=	Small Mothers Against Decapentaplegic
SOD	=	Superoxide Dismutase 1
SLE	=	Systemic Lupus Erythematosus
TCR	=	T cell Receptor
TEC	=	Thymic Epithelial Cell
TGF	=	Tumor Growth Factor
TNF	=	Tumor Necrosis Factor
Wnt	=	Wingless

Appendix II

List of publications

I. Pignata C, Amorosi S, Giovannini M, Russo I, Matrecano E, Busiello R. L'apoptosi nella fisiologia della risposta immune e sue alterazioni nella patogenesi di quadri di patologia complessa.(Prosp Ped. 2006;36:147-156)

II. Adriani M, Garbi C, Amodio G, Russo I, Giovannini M, Amorosi S, Matrecano E, Cosentini E, Candotti F, Pignata C. Functional interaction of common γ-chain and growth hormone receptor signaling apparatus.(J Immunol. 2006;177:6889-6895)

III. Amorosi S. Potente effetto anti-oncogenico del silenziamento molecolare della catena gamma. XI Congresso Internazionale "Incontri Pediatrici Normanni :I bambini nell'europa di oggi e di domani", Aversa (Italy), November 2007.

IV. Pignata C., Amorosi S., Russo I., Capalbo D., Lettiero T., Adriani M., Salerno M.C. Shared signalling pathways between endocrine and immune system receptors: the model of gamma chain.(Curr Sign Trasd Ther. 2008;3:206-214)

V. Broccoletti T., Del Giudice E., Amorosi S., Russo I., Di Bonito M., Imperati F., Romano A., Pignata C. Steroid-induced improvement of neurological signs in ataxia-teleangiectasia patients.(Eur J Neurol. 2008;15:223-228).

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VII. Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., Palamaro L., Marsilio Adriani, Vigliano I., Pignata C. The common γ chain provides spontaneous and GH-dependent cell cycle progression, related to its cellular amount.(J Immunol. 2009;82:3304-3309)

VIII. Russo I., Cosentino C., Broccoletti T., Amorosi S., Cirillo E., Aloj G., Fusco A., Costanzo V., Pignata C. In Ataxia-Teleangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to anti-oxidative capacity.(Eur J Neurol. 2009;16:755-759)

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1. Amorosi S., Adriani M., Russo I., Matrecano E., Giovannini M., Amodio G., Pignata C. Evaluation of a functional interaction between the common gamma chain and growth hormone receptor using a neutralization assay. XIIth Meeting of the European Society For Immunodeficiencies IXth Meeting of the International Patients Organisation For Primary Immunodeficiencies VIIth Meeting of the International Nursing Group For Immunodeficiencies. Budapest, October 4-7, 2006.

2. Giovannini M., Adriani M., Matrecano E., Garbi C., Amorosi S., Russo I., Candotti F., Pignata C. GH-induced signaling and STAT5b nuclear translocation in control or X-SCID EBV cell lines. XIIth Meeting of the European Society For Immunodeficiencies IXth Meeting of the International Patients Organisation For Primary Immunodeficiencies VIIth Meeting of the International Nursing Group For Immunodeficiencies, Budapest, October 4-7, 2006.

3. Russo I., Matrecano E., Crescenzo C., Fusco A., Amorosi S., Broccoletti T., Vecchione E., Dianzani U., Pignata C. Identification and functional characterization of patients with autoimmunity associated with functional immunodeficiency. XIIth Meeting of the European Society For Immunodeficiencies IXth Meeting of the International Patients Organisation For Primary Immunodeficiencies VIIth Meeting of the International Nursing Group For Immunodeficiencies, Budapest, October 4-7, 2006.

4. Amodio G., Adriani M., Amorosi S., Matrecano E., Giovannini M., Candotti F., Pignata C. Transduction of γ⁻X-SCID EBV cells with wild type γc gene restores GH-induced STAT5 phosphorylation. XIIth Meeting of the European Society For Immunodeficiencies IXth Meeting of the International Patients Organisation For Primary Immunodeficiencies VIIth Meeting of the International Nursing Group For Immunodeficiencies, Budapest, October 4-7, 2006.

5. Amodio G., Amorosi S., Broccoletti T., Benedetto G., Russo I., Crescenzo C., Pignata C. Analisi della trasduzione del segnale via GHR in linee cellulari EBV di pazienti affetti da X-SCID e controlli. X Congresso Internazionale "Incontri Pediatrici Normanni", Aversa (Italy), Novembre 24-25, 2006.

6. Pignata C., Russo I., Amorosi S., Broccoletti T. Nuove e vecchie immunodeficienze. X Congresso Internazionale "Incontri Pediatrici Normanni", Aversa (Italy), Novembre 24-25, 2006.

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8. Amorosi S., Russo I., Di Bonito M., Vecchione E., Pignata C. Rapporto tra SCID-X1 e mutazione della catena γ e funzionalità del GHR. IX Giornate "Giovani" di Prospettive in Pediatria, Naples, February 15-17, 2007.

9. Broccoletti T., Del Giudice E., Amorosi S., Russo I., Di Bonito M., Secchione E., Strisciuglio C., Romano A., Pignata C. Oral betamethasone improves neurological symptoms in Ataxia-Teleangiectasia. 48th Annual meeting of the Europen Society for Paediatric Research , Prague, October 6-8, 2007

10. Russo I., Amorosi S., Vitiello L.,Capalbo D., Broccoletti T., Salerno M.C., Pignata C. Analisi dell' alterazione dei meccanismi di tolleranza periferica associata ad alta variabilità fenotipica. XI Congresso Internazionale "Incontri Pediatrici Normanni :I bambini nell'europa di oggi e di domani", Aversa (Italy), November 2007.

11. Russo I., Cosentino C., Amorosi S., Broccoletti T., Aloj G., Cirillo E., Fusco A., Costanzo V., Pignata C. In Atassia-Teleangectasia betamethasone response correlates inversely to cerebellar atrophyand directly to antioxidative capacity. Day of Immunology. Primary Immunodeficiencies: emerging challenges. Napoli 29 Aprile 2008

12. Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., Palamaro L., Adriani M., Vigliano I., Pignata C. The common γ chain provides spontaneous and GH-dependent cell cycle progression, related to its cellular amount. Day of Immunology. Primary Immunodeficiencies: emerging challenges. Napoli 29 Aprile 2008

13. T. Broccoletti, S. Amorosi, E. Cirillo, G. Aloj, F. Santamaria, F. Fusco, MV. Ursini, A. Durandy, C. Pignata. A novel form of non X-linked Hyper-IgM syndrome associated with lymphoma development. Day of Immunology. Primary Immunodeficiencies: emerging challenges. Napoli 29 Aprile 2008

14. Broccoletti T., Amorosi S., Cirillo E., Aloj G., Santamaria F., Fusco F., Valeria Ursini, Durandy A., Pignata C. A novel form of non X-linked Hyper-IgM syndrome associated with lymphoma development. Casi clinici Sperimentali del Dipartimento di Pediatria Università "Federico II", Napoli 24 Aprile 2008

15. Amorosi S., Capalbo D., Vitiello L., Fusco A., Ursini V., Salerno M., Pignata C. High intrafamilial variability in APECED syndrome: a peripheral tolerance study. Casi clinici Sperimentali del Dipartimento di Pediatria Università "Federico II", Napoli 29 Maggio 2008.

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17. Amorosi S., Russo I., Amodio G., Garbi C., Vitello L., Palamaro L., Vigliano I., Pignata C.g-chain provides a spontaneous and GH-dependent signal for cell cycle progression related to its cellular amount. Boston (USA), Focis Annual Meeting, June 5-9 2008.

18. Broccoletti T., Amorosi S., Di Bonito M., Cirillo E., Aloj G., Fusco F., Ursini MV., Durandy A., Pignata C. A novel form of Hyper-IgM sindrome. Boston (USA), Focis Annual Meeting, June 5-9 2008.

19. Amorosi S., Capalbo D., Fusco A., Vitello L., Buzi F., Salerno M.C Pignata C. High intrafamiliar variability in APECED: study of the peripheral tolerance XIIIth Meeting for the European Society for Immunodeficiencies. Budapest 16-19 October 2008

20. Broccoletti T., Amorosi S., Russo I., V Urini V., Buffardi S., Carsetti R., Durandy A., Pignata C. Novel Class Switch Recombination defect reminiscent of ICV and evolving in lymphoma. XIIIth Meeting for the European Society for Immunodeficiencies. Budapest 16-19 October 2008

21. Russo I., Cosentino C., Broccoletti T., Amorosi S., Fusco A., Cirillo E., Aloj G., Costanzo V., Pignata C. In Ataxia-Teleangectasia betamethasone response correlates inverselyto cerebellar atrophy and directly to antioxidative capacity. XIIIth Meeting for the European Society for Immunodeficiencies. Budapest 16-19 October 2008.

22. Amorosi S, Guarino V, Fusco A, Vigliano I, Gorrese M, Del Vecchio L, Ambrosio L, Pignata C., Comparazione del blocco ontogenetico T nei due modelli umani di ATIMIA NUDE/SCID e DIGEORGE e allestimento di uno "Scaffold" tridimensionale per la generazione in vitro di cellule T da precursori ematopoietici in assenza di timo. Relazione alla Giornata di Ricerca del Dipartimento di Pediatria. Aula del Dipartimento di Pediatria. Napoli 17 aprile 2009

23. Fusco A, Amorosi S, Vigliano I, Vitiello L, Racioppi L, Gorrese M, Del Vecchio L, Pignata C., La mutazione del gene FOXN1 associata al fenotipo NUDE/SCID previene completamente il differenziamento dei linfociti CD4, ma non dei CD8. Relazione alla Giornata di Ricerca del Dipartimento di Pediatria. Aula del Dipartimento di Pediatria. Napoli 17 aprile 2009

24. Lettiero T, Amorosi S, De Leonibus C, Amoresano A, Pucci P, Urini MV, Pignata C, Salerno M Insensibilità all'ormone della crescita e grave bassa statura: studio biochimico e molecolare. Relazione alla Giornata di Ricerca del Dipartimento di Pediatria. Aula del Dipartimento di Pediatria. Napoli 17 aprile 2009

25. Amorosi S, Fusco A, Vigliano I, Gorrese M, Del Vecchio L, , Pignata C., Characterization of the T-cell ontogeny defect in the human athymic models of the Nude/SCID and DiGeorge syndromes. Day of

Immunologo “Mucosa associated immune responses: between tolerance and inflammation”. Avellino 29 aprile 2009. (Selected oral presentation)

During my PhD, I, also, participated to several National or International Scientific Meetings, in two of which I received the following Travel Awards:

2007. Award “5° Premio Provincia di Caserta” with the work entitled: “Potente effetto anti-oncogenico del silenziamento molecolare della catena gamma”. XI Congresso Internazionale “Incontri Pediatrici Normanni :I bambini nell'europa di oggi e di domani”, Aversa (Italy), November 2007.

2008. Travel Award with the work entitled: “γ-chain provides a spontaneous and GH-dependent signal for cell cycle progression related to its cellular amount”, Boston (USA), Focis Annual Meeting, June 5-9 2008.

Appendix III

Grant Proposals

During my PhD, I, also, contributed to the writing of several Grant Proposals, among which:

1. Programma di Ricerca Scientifica di Rilevante Interesse Nazionale (Prin) annualità 2006, Implication of mutations of the gamma chain gene, responsable for the human SCID-X1 phenotype, on GH receptor-induced cell signaling.
2. Telethon Application 2007, Implication of gamma chain mutation, responsible for SCID-X1, on growth hormone receptor signaling and functionality.
3. Bando Giovani Ricercatori 2007, Potenziale ruolo oncogenico della catena gamma.
4. Telethon Application 2008, Lymphoproliferation and gene therapy for X−SCID: studies on the role of gamma chain in cell cycle progression and cellular homeostasis in normal and IL2RG transduced cells.
5. Telethon Application 2009, Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes.
6. Programma per la Ricerca Sanitaria 2008: attività di Ricerca sulle Malattie Rare, Potential oncogenic role of the X-SCID gamma chain gene, as Scientific Coordinator of the participating Unit 1.
7. Progetti di Ricerca Scientifica 2008 Finanziabili ai sensi della L.R. N.5 del 28.03.2002, Realizzazione di uno "scaffold" tridimensionale di

policaprolattone per la generazione in vitro di linfociti T maturi a partire da cellule staminali.

8. Programma di Ricerca Scientifica di Rilevante Interesse Nazionale (Prin) annualità 2008, Study of T-cell ontogeny in human models of athymia and autoimmunity: Setting up of a 3-dimensional matrix to support the in vitro production of T-cell and Treg from hematopoietic precursor cells.

9. Bando Ricerca Scientifica in ambito biomedico Fondazione Cariplo 2009, Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood.

10. AIP (Associazione Italiana di Pediatria) Application 2009, Clinical and molecular characterization of pediatric patients affected with APECED: identification of functional and genetic factors influencing the phenotype expression of the disease.