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CONGENITAL IMMUNODEFICIENCIES AND GH/IGF-I AXIS FUNCTION: CROSS-TALK BETWEEN COMPLEX SYSTEMS

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To my Family and Rosy

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BACKGROUND AND AIM OF THE PROJECT

Up to fifty years ago the only property of the lymphocytes known was that they are mobile. In the 1953 Arnold Rich, Baxley Professor of Pathology, concluded talking about the “small lymphocytes” than “literary nothing of importance is known regarding the properties of these small cells” (1). At that time there was little evidence to convince anyone that small lymphocytes had immunological functions. In 1959, Burnet wrote than: “An objective survey of the facts could well lead to the conclusion that there was no evidence of immunological activity in small lymphocytes” (2). The view that Burnet expressed in the 1959 had to be updated a few years later, when an immunological role for small lymphocytes was unequivocally demonstrated by testing the ability of small lymphocytes from parental-strain rats to cause graft versus host (GVH) after injection into adult F₁ hybrid rats. Such inocula induced a vicious reaction in the recipients, with progressive wasting and death after a time depended on the cell dose. For the first time was showed, that during the early stages of the resulting GVH reaction, the donor small lymphocytes enlarged and began dividing (3, 4).

Only in the 1962, for the first time a role of small lymphocytes in the immune response was suggested (4). The first persuasive evidence for the existence of two classes of lymphocytes came in the 1968 from the work of Mitchell and Miller (5) and the idea that thymus-derived (T) lymphocytes “helped” the development of marrow (B) lymphocytes was proposed for the first time by Mitchison in the 1971 (6).

Since these early studies, the lymphocyte has risen to become one of the most studied of all mammalian cells. Tremendous progresses achieved in developmental, cellular and molecular immunology has been achieved in the past 20 years, largely due to studies using the mouse model system and the arrival of molecular genetics.

The generation in 1989, for the first time of a knockout animal by Thompson and colleagues (7), revolutionized the studies of mammalian physiology, and immunologist

were among the first to take advantage of the new approach. The technology was rapidly and widely adopted and hundreds of genetically designed knockout mice are now available to help us understand the roles of specific gene in development of the immune system as well as in disease situation such as autoimmunity, infections and graft rejections (8; <http://www.informatics.jax.org/>).

Despite the abundant knowledge that is now available in the immunology field, surprising little is known, however, about immunity to infection. We know about the generation of antibody diversity, the structure of surface molecules of lymphocyte subsets, and the role of the major histocompatibility complex (MHC) in cell cooperation and antigen recognition. However, we know little about the function of most of these molecules and cell subsets.

To study immunity to infection is undeniable difficult due to the high complexity of the host-environment relationship and the interaction between different cells and components of the organism. In this contest also the forward-genetic studies in mice only in few case have proven to be have clinical relevant implication. The difficulty in translating genetic discoveries made in mice into real clinical benefits might reflect intrinsic molecular differences between species, due to phylogenical distances that separate human and mice (9). Nevertheless, mice studies provided us a huge number of candidate genes for human studies.

Immunodeficiency disorders are a diverse group of illnesses that increase susceptibility to infection (10, 11). Primary immunodeficiencies, naturally occurring defects of the immune system, were not identified until after the introduction of antibiotics because of high morbidity and mortality of those affected early in life (10). They are mostly congenital and hereditary, with broad clinical presentations that reflect the complexity of the immune system and the underlying genetic defects. Combined immunodeficiencies (CIDs) are a heterogeneous group of disorders characterized by defects in T-cell development and/or function, associated with abnormal function or

development of B and NK cells (12). Patient with defects that involve T cells do not have adequate cellular immune responses and are predisposed to developing opportunistic infections (13). Severe combined immunodeficiencies (SCID) represent the most severe form of CIDs, and have an overall frequency of approximately 1 in 75,000 births. SCID conditions share the characteristic of a profound block in T cell differentiation (14, 15) and are lethal within the first year of life because of absent T cell-mediated immunity. Most forms of SCID are associated with molecular alterations of genes selectively expressed in hematopoietic cells and implicated in cell differentiation/activation process. The only SCID condition caused by an alteration in a gene not expressed in the hematopoietic compartment is the human Nude/SCID phenotype, which is caused instead by a thymic defect (16-18) (see Chapter II of this Thesis). The molecular defects in many case of SCID have yet to be identified.

This thesis reports the results I obtained during the Dottorato di Ricerca in “Accrescimento, Sviluppo e Riproduzione dell’uomo” (XVIII Cycle) from 2002-2006. During the past 4 years, my research has been focused in the molecular and functional characterization of patients with primary immunodeficiencies.

Based upon previous studies performed in Prof. Pignata’s laboratory, which characterized an atypic case of X-linked SCID phenotype associated with growth hormone hyporesponsiveness (19) and identified the first case of human Nude/SCID phenotype (17), I set out to define the biochemical and molecular mechanisms of the immune response regulation and to elucidate the basis of the interplay between the IS and other systems of the organism by evaluating the perturbations of this interplay in patients with congenital immunodeficiency (CID).

In particular the following three lines were developed:

- Role of the interaction between Endocrine System and Immune System in the physiology of the immune response in particular functional

interactions between common gamma chain and Growth Hormone Receptor signaling elements were examined.

- Molecular and clinical characterization of the human Nude/SCID phenotype and identification of clinical signs suggesting a previously unappreciated functional role of FOXP1 transcription factor in the development and differentiation of the central nervous system.
- Role of the Interleukin-12/Interleukin-12R (IL-12/IL12R) axis in diseases associated with predominant T-helper 2 (Th2) activity

CHAPTER 1

EVIDENCES OF A PREVIOUSLY UNAPPRECIATED FUNCTIONAL INTERACTIONS BETWEEN COMMON GAMMA CHAIN AND GROWTH HORMONE RECEPTOR SIGNALING ELEMENTS

Introduction

The most common form of Severe combined immunodeficiency accounting for approximately half of all cases with an estimated incidence of 1:150000 to 1:200000 live births is the X-linked SCID (X-SCID) (20, 21). This form of SCID is characterized by 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-cells differentiation pathway in this disease (22). 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 (23, 24). These observations and data from X-chromosome inactivation studies in obligate carriers, showing a skewed pattern of inactivation in B cells, as well as in T and NK cells, suggested that an intrinsic defect of the B-cell compartment is also present in this form of SCID (25, 26).

Atypical cases of X-SCID were reported, including forms with progressive loss of T-cell number and function, leading to death during childhood (27, 28). A unique patient, in whom a reverse mutation in an early T-cell progenitor, with normal amounts of circulating T cells, was also described (29). In many cases the clinical and phenotypic heterogeneity of X-SCID remain poorly understood.

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 cytokines receptors critical for the development and function of lymphocytes such as Interleukin- (IL-) 2, IL-4, IL-7, IL-9, IL-15 and IL-21 (21). Cytokines regulate cellular activation, proliferation, differentiation, and survival. A common feature for cytokines is that their receptors lack intrinsic tyrosine kinase activity and that they use the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway to mediate gene activation or repression.

The JAK/STAT pathway is one of a handful of pleiotropic cascades used to transduce a multitude of signals for development and homeostasis in mammals (30). JAKs are cytoplasmic tyrosine kinases pre-associated with the intracellular domains of cytokine receptors. The binding of the ligand to the specific receptors results in dimerization of receptor subunits, thus increasing the local concentration of JAKs bringing these kinases into close proximity, allowing their trans-phosphorylation and activation. The activated JAKs initiate the signal transduction cascade by phosphorylation of tyrosine motifs present in receptor cytoplasmic domains and in receptor-associated proteins. Phosphotyrosine-containing motifs in receptor cytoplasmic domains act as docking sites for many signaling proteins, including STATs. STAT proteins are also phosphorylated on a conserved tyrosine residue. Tyrosine phosphorylation induces STATs activation resulting in dissociation from the receptor, dimerization and acquisition of high-affinity DNA-binding activity. After activation STATs translocate to the nucleus, where they bind to gene promoters and activate transcription (21, 30-32).

There are four members JAKs (JAK1-3 and TYK2) and seven STAT proteins (STAT1-4, STAT5a, STATb and STAT6) (32). All γ_c -containing cytokine receptors can activate JAK1 and JAK3 (33-35). Although many cytokines can induce activation

of JAK1, only cytokines with a γ_c -dependent receptor can induce activation of JAK3 (21). JAK3 is selectively associated with γ_c (36). STAT 1, STAT3 and STAT5 are activated after binding of the specific ligand to γ_c -dependent cytokine receptors. Whereas JAK3 can only phosphorylate STAT3 and STAT5, JAK1 can phosphorylate STAT1, STAT3, and STAT5, but only in the presence of an activated JAK3 (37). This would indicate that γ_c -associated JAK3 activates JAK1 which is then able to induce activation of the target STATs molecules (38).

To our knowledge, an extrahematopoietic role of γ_c has not yet been demonstrated, although the abundance of the protein in non-hematopoietic cells would imply additional functions for this element (39, 40).

We previously reported on a patient affected with a X-linked Severe Combined Immunodeficiency (X-SCID) who received a bone marrow transplantation (BMT) 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 were demonstrated.

The GHR was the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for Erythropoietin (EPO), Granulocyte colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukins- (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 Janus associated kinase 2 represents the predominant non receptor tyrosine kinase required for the initiation of GH signal transduction upon ligand binding to the receptor (41, 42). However, GH also stimulates tyrosine phosphorylation of JAK1 (43, 44) and JAK3 (34) in certain cell lines. Signal transduction through GHR also involves a wide array of molecules, such as STAT1, 3 and 5, Extracellular regulated kinase (ERK) 1 and 2 and

Phosphatidylinositol-3 kinase-protein kinase B (42). 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-I (IGF-I), which mediates many of the GH biological functions (45-47). 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 (19).

Since we hypothesized a role for the γ_c chain in GHR signaling, in this study, we evaluate the functional interaction between GHR and the common γ element in either freshly isolated or Epstein-Barr virus (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 STAT5b protein.

Materials and Methods

Reagents

Recombinant human growth hormone (rGH) was obtained from Serono (Saizer® 4). The enhanced chemiluminescence kit (ECL) was purchased from Amersham Biosciences. The antibodies 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 monoclonal-antibodies anti-phosphotyrosine were purchased from Santa Cruz Biotechnology (Santa Cruz). The antibody 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 Becton Dickinson. Epidermal Growth Factor was purchased from BD Bioscience and used at the concentration of 100 ng/ml. Acrylamide and bisacrylamide were from Gibco. Prestained molecular weight standards were from BIORAD. Except where noted, other reagents were of either reagent or molecular biological grade from Sigma.

EBV stock preparation

EBV-transformed B95-8 marmoset cell line were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS). The cultures are maintained for 12-15 days. After centrifugation for 5 min at 1500rpm, the supernatant was filtered through a 0.22- μ m membrane (Millipore) and stored at 4 °C until use.

Cells separation

Mononuclear cells (PBMC) were obtained from 4 X-SCID patients in which γ c mutations led to the absence of protein expression and normal donors heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Briefly, after dilution 1 to 1 with PBS pH 7.4 blood samples are carefully laid on Ficoll-Hypaque at a 2 to 1 ratio. After centrifugation at 1500 rpm for 30 min at 18-20 °C in a SORVALL® Legend RT centrifuge, the upper layer of plasma was drawn off and the lymphocyte layer transferred to clean centrifuge tubes. Lymphocytes were then suspended in 3 volumes of PBS, tubes centrifuged at 1500 rpm for 15 min at 18-20 °C and the supernatant removed. The wash step was repeated two times. Cells were suspended in a known volume of complete media (RPMI 1640 media supplemented with 10% FBS, 2 mmol/L L-glutamine, and 50 µg/ml gentamycin, all from Gibco) and counted using a hemocytometer (Hausser Scientific).

NIH 3T3 fibroblasts were used in a few experiments.

Immortalization of lymphocytes

Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by Epstein-Barr virus immortalization of patients and controls PBMC using standard procedures (48). Briefly, immortalization was achieved by mixing 3×10^6 PBMC of each subject suspended in 3 ml of RPMI complete medium with 1 ml of active EBV supernatant in the presence of 2 µg/ml cyclosporin A (Sigma) in 14 ml Polypropylene round-bottom tubes (Falcon). After 24 h cells were washed two times with warm PBS, counted and put in culture in a 96 wells tissue plate (10^5 cells/well) in fresh media containing 1 µg/ml cyclosporin A. Continuous cellular passages were made every 3 or 4 days (twice per week) by exchanging an equal volume of cultivating cell suspension with fresh medium. Growing cells were moved in 25 cm² tissue culture flask (Corning).

Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Gibco), and 50 µg/ml gentamycin (Gibco), and cultured at 37°C, 5% CO₂ or frozen in 10% dimethylsulfoxide (DMSO) and kept in liquid nitrogen..

Transduction of Lymphocytes

pGC2Rγ retroviral vector (49) was used to transduce X-SCID BCLs with WT γc as previously described (50).

The transduction procedure was initiated by performing a phosphate depletion step for 3-12 hr at 37°C prior to the transduction start point by pelleting the cells, washing the cells in the RPMI 1640 phosphate-free medium, and resuspending the cells at 1 x 10⁶/ml (2 ml per well, in 6-well plates) in RPMI 1640 phosphate-free medium supplemented with 5% heat-inactivated FBS and rIL-2 at 200 U/ml. The phosphate-starved cells were subsequently exposed to the retroviral vector by the addition of supplemented supernatant, followed by centrifugation at 32°C for 60 min and incubation for 12 hr at 32°C in a 5% CO₂ incubator. At the end of the transduction, the lymphocytes were pelleted, washed, and cultured at 1 x 10⁶ cells per ml in AIM-V media supplemented with 5% fetal bovine serum and rIL-2 at 200 units/ml. Lymphocyte viability and number were determined using trypan blue exclusion.

Transduced cells were selected in the neomycin-analogue G418 (Cellgro®).

Proliferative assay

BCLs (1 X 10⁵ cell/200 µl well) were cultured triplicate in 96-well U-bottomed microtiter plates (Falcon®, Becton Dickinson) 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 [^3H]thymidine (Amersham International) 8 h before harvesting (51). 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 the γc chain (CD132) was detected using specific rat antibodies phycoerythrin-(PE-) conjugated (Becton-Dickinson) by direct immunofluorescence. After washing in PBS, 1×10^6 cells were suspended in 100 ml of staining buffer (PBS + 2% FBS) and incubated for 10 min on ice in presence of 2 μl unlabeled mouse IgG. Without washing 2 μl of PE-conjugated rat isotype control antibodies (Becton-Dickinson) or 2 μl of PE-conjugated anti-CD132 specific antibodies were add to the cells. After 30 min of incubation on ice all samples were washed in PBS and acquired on the LSRII flow cytometer (Becton-Dickinson) using DIVA software for acquisition and FlowJo software (TreeStar) for the data analysis.

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

Cell stimulation and protein extraction

Prior to 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 phosphate-buffer saline (PBS; BioWhittaker) followed by solubilization in 100 μ l of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na_3VO_4), 5 μ g/ml leupeptin and 5 μ g/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.* (52) and were subsequently mixed with sample buffer.

Western blot

Immunoblotting using phosphotyrosine monoclonal antibody was performed as previously reported (19). Immunoblotting using specific antibody was performed according to the vendors protocols. In brief, protein samples separated by SDS-PAGE were transferred onto Mixed Cellulose Esters membranes (Immobilon-NC Mixed Cellulose Esters 0.45 μ m; Millipore). The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 10% Bovin Serum Albumin (BSA) in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 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 antibody. The membrane was then washed three times and an appropriate IgG horseradish peroxidase-conjugated secondary antibody 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 4 rinses of 5 min in PBS, the cells were centrifuged in a Shandon Cytospin III (Histotronix Inc.) onto 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 antibodies against STAT5b diluted 1:100 in PBS containing 1% BSA. After 4 washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with a 1:200 dilution of Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Pierce Biotechnology Inc.) 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 lines proliferation *in vitro*, its effect being direct and not mediated by IGF-I (51). 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. 1.1A, GH enhanced proliferation of BCLs of control subjects in a dose-dependent fashion. Significant enhancement of [³H]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 didn't 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 on control's and patient's cells (Fig. 1.1B). No difference was found in the mean fluorescence intensity (130.99 ± 28.19 vs 139.88 ± 33.49 in patients and controls, respectively; $p = \text{NS}$) 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. 1.1C, the neutralizing MAB inhibited by 64% the proliferative response to GH. A non specific effect of the Ab was ruled out, since the IgG1 anti-CD3 isotype matched Ab was ineffective in inhibiting cell proliferation.

To define whether PI3 kinase had a role on GH-induced cell proliferation of BCLs to GH, the kinase inhibitor wortmannin was used. As shown in Fig. 1.1D, no inhibitory

effect was appreciable. By contrast, in the positive control wartmannin was able to inhibit fibroblast proliferation to EGF by 85%.

To ascertain whether γc was linked to GHR, we then assessed by confocal microscopy the plasma membrane expression of these 2 molecules. As shown in Fig. 1.1D, by indirect immunofluorescence using specific antibodies, as previously detailed, co-localization of γc and GHR was observed on the cell surface of normal BCL cells.

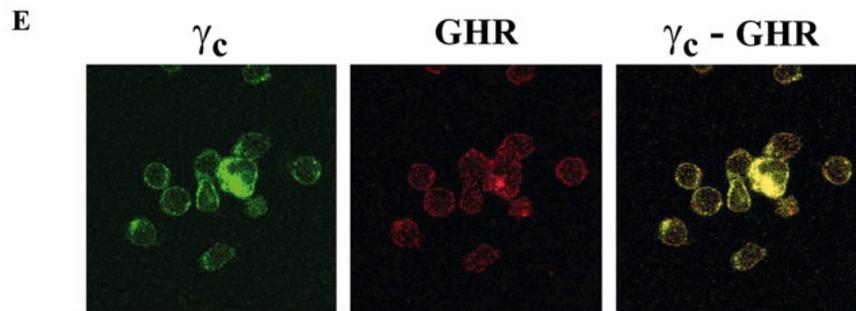
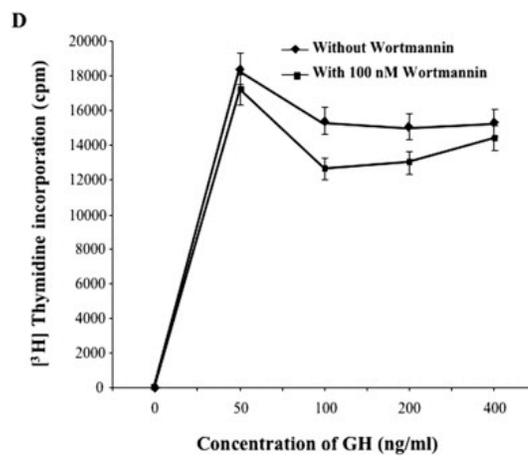
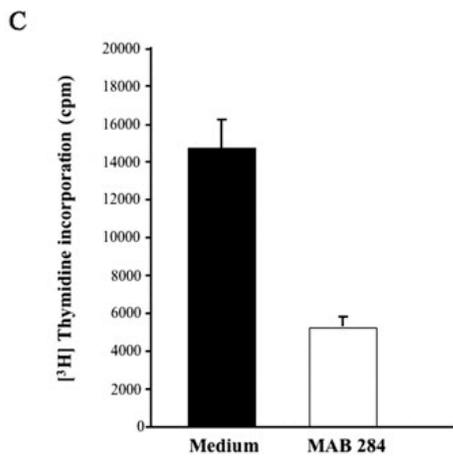
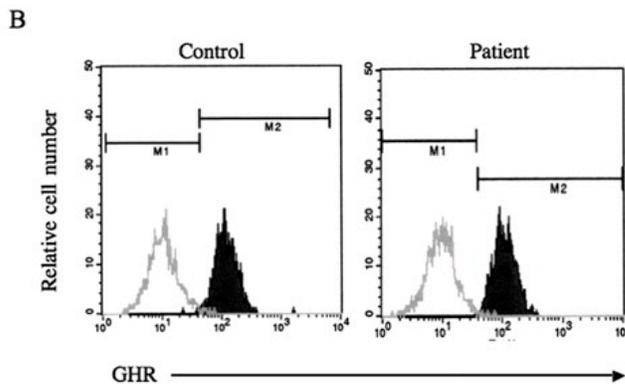
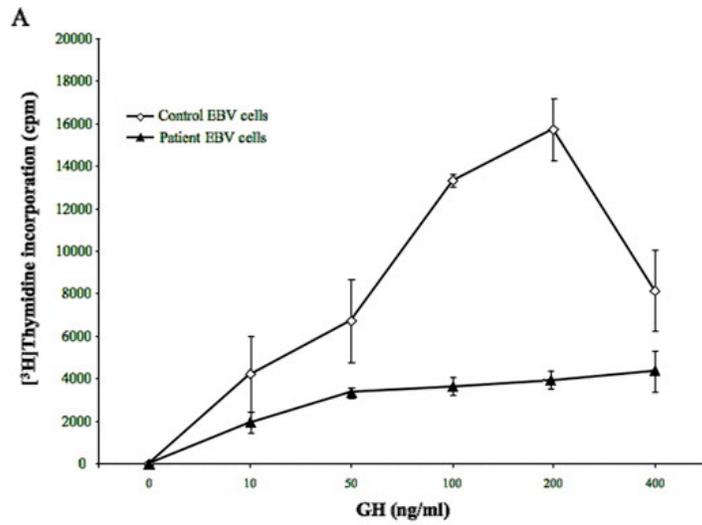


Figure 1.1. In vitro effect of GH stimulation on proliferation of X-SCID patients' and controls' EBV cell lines and membrane localization of GHR and γ c. (A) Lymphoblastoid cell lines (BCLs) were generated by Epstein-Barr virus immortalization of patients' and controls' PBMC using standard procedures and cultured in the presence of various concentrations of GH for 4 days. Cultures were pulsed with [³H]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 (closed bars) or with the neutralizing MAB 284 (open bars) 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 pre-treated 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*, Gamma chain co-localizes with GHR. Normal BCL cells were double labelled with anti- γ c (left) and anti-GHR (center) antibodies. Confocal microscopic analysis indicates a plasma membrane localization for both molecules. The yellow colour in the merge, (showed on the right), indicates areas of co-localization 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 analysing the number and the timing of the proteins phosphorylated on tyrosine residues. Fig. 1.2 illustrates a representative immunoblot with anti-phosphotyrosine antibodies of whole cell lysates from patient's and control's BCLs following stimulation with GH for 5, 15 or 30 min. In contrast to what observed in control cells, in patients GH stimulation failed to induce phosphorylation of proteins approximately of 90 kDa, presumably corresponding to STATs 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).

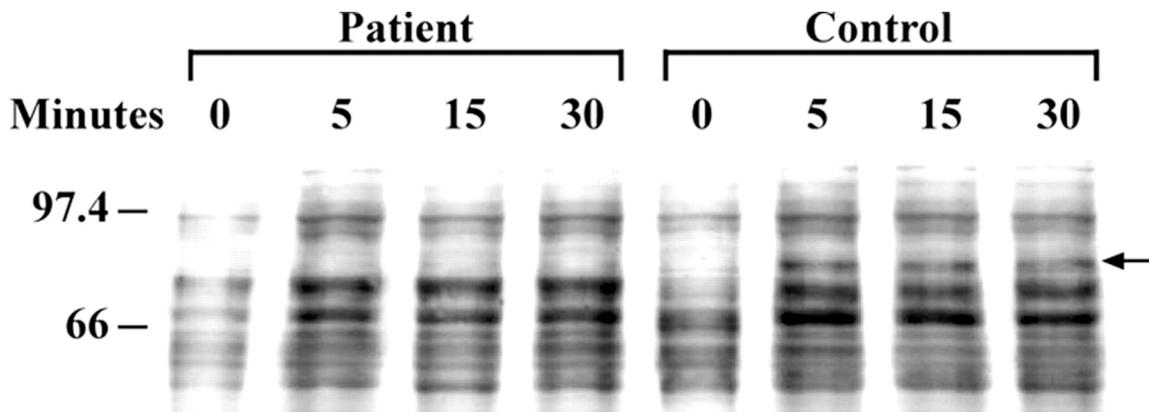


Figure 1.2. Pattern of protein tyrosine phosphorylation induced through GHR engagement. BCLs from X-SCID patients and healthy subjects were starved of serum for 8-12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. Stimulation was stopped with cold PBS and BCLs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-phosphotyrosine antibodies.

GHR signal transduction pattern

The 3 main signal modules by which signal transduction through GHR occurs involve Mitogen-activated protein kinases/ERK1 and 2, JAK2, STAT1, STAT3 and STAT5 and the Phosphatidylinositol-3 kinase-protein kinase B signalling (42).

To evaluate whether the γc was involved in the GHR signaling events, we first focused on STAT5 molecule. As shown in Fig. 1.3, in control's BCLs, tyrosine phosphorylation of STAT5 was evident, with a peak of activity observed between 5 and 15 min after GH stimulation. By contrast, in patient's BCLs 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. 1.4, A, B, C, and D). No difference in the phosphorylation events between patient' and control' BCLs was appreciable. As shown, the expression of the molecules was comparable in control and patient cells.

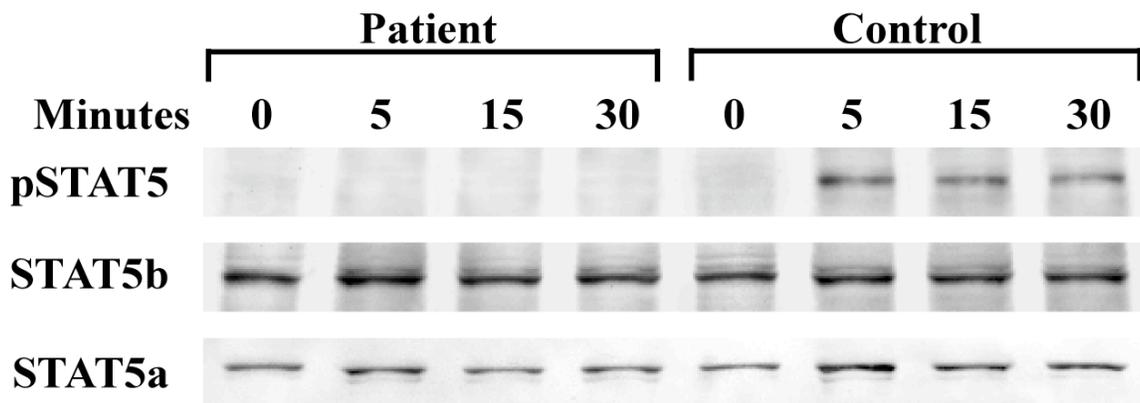


Figure 1.3. STAT5 phosphorylation induced through GHR stimulation. rGH stimulation failed to induce STAT5 tyrosine phosphorylation in γ c negative BCLs. BCLs from X-SCID patients and healthy subjects were starved of serum for 8-12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. After SDS-PAGE and Western blot, membranes were incubated with anti-pSTAT5, anti-STAT5b or anti-STAT5a antibodies.

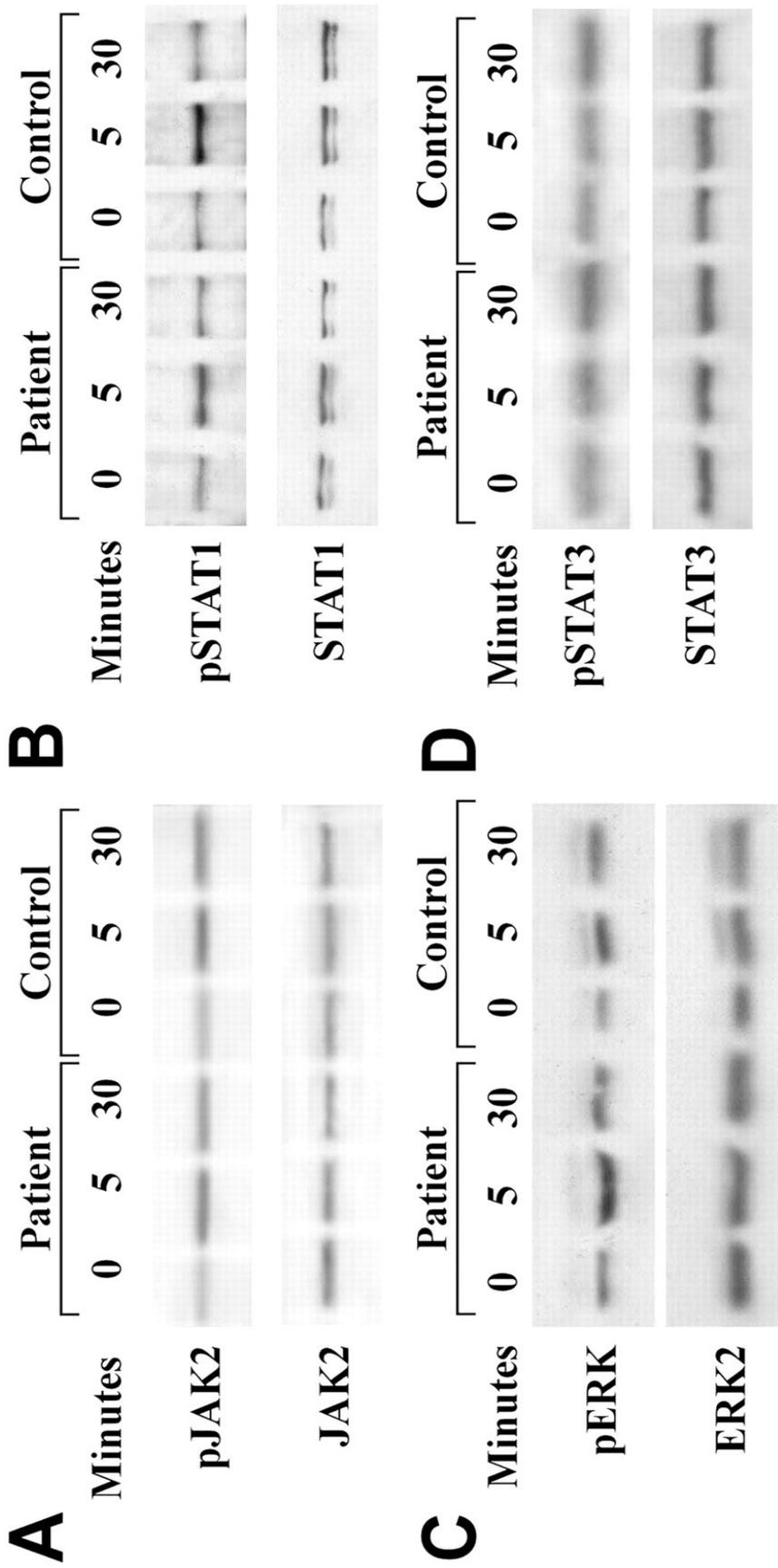


Figure 1.4. Phosphorylation events induced through GHR stimulation. BCLs from X-SCID patients and healthy subjects, starved of serum for 8-12 h, were stimulated for the indicated time with rGH at the concentration of 500 ng/ml. After SDS-PAGE and Western blot, membranes were incubated with (A) anti-pJAK2 or anti-JAK2, (B) anti-pSTAT1 or anti-STAT1, (C) anti-pERK or anti-ERK2, (D) anti-pSTAT3 or anti-STAT3.

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 (53, 54). 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.

Patients and controls BCLs were stimulated with GH for 30 min, fixed and incubated with antiserum against STAT5b. As shown in Fig. 1.5, 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 control's BCLs, 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. 1.6, in controls 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 the 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.

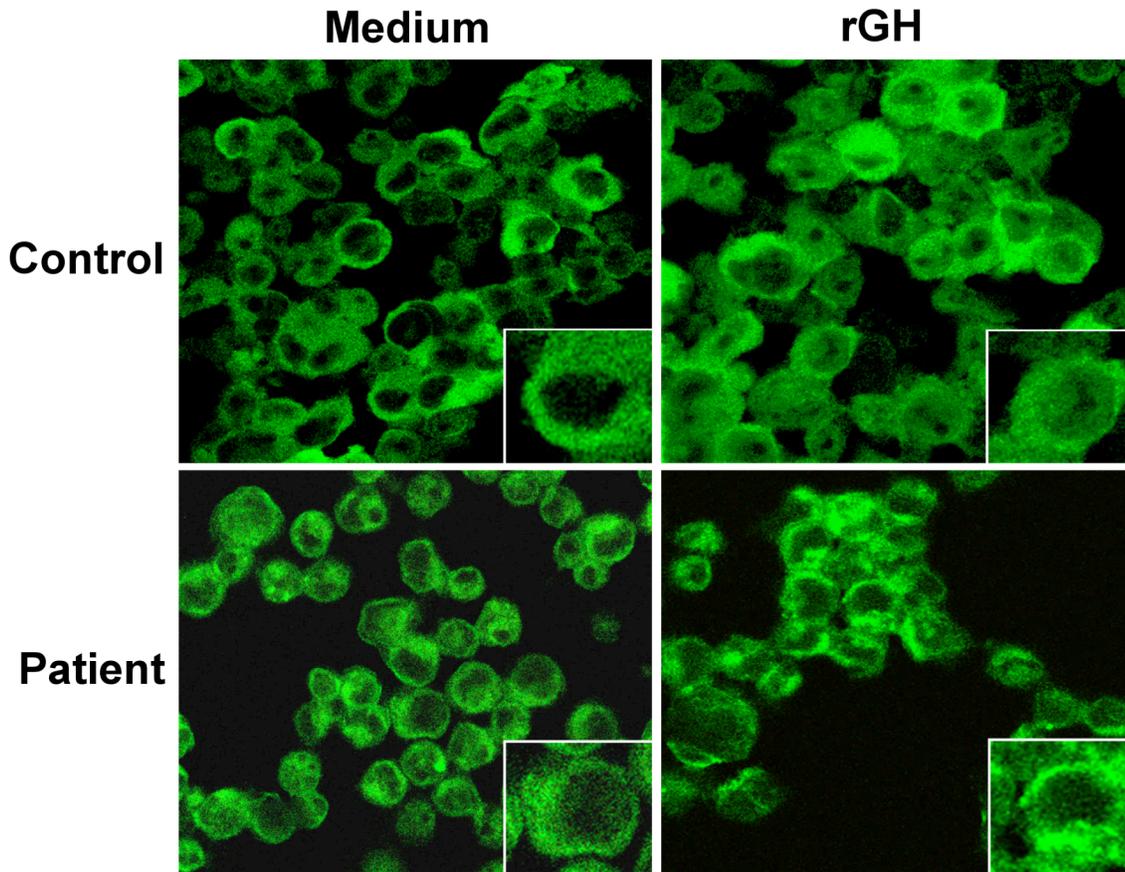


Figure 1.5. STAT5b subcellular localization. X-SCID patients and healthy subjects control cells were cultured in the absence or presence of 500 ng/ml rGH for 30 min at 37°C. Unstimulated or stimulated cells were analyzed by Confocal microscopy for STAT5b (green) distribution in the cell, focusing particularly on whether this protein was present in the nuclei.

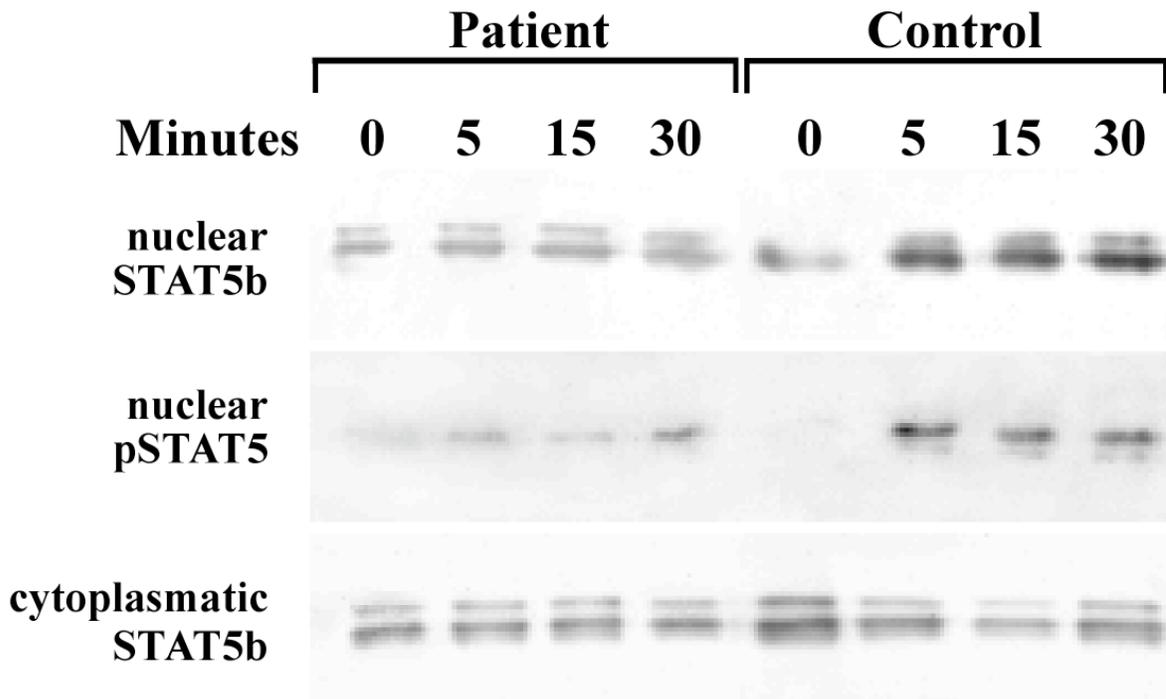


Figure 1.6. Nuclear fraction of the overall STAT5b amount and of the phosphorylated form of STAT5 in resting or rGH stimulated BCLs. Patient and control BCLs were stimulated with rGH (500 ng/ml) or medium alone at 37°C for the indicated time. Stimulation was stopped with cold PBS and nuclei were isolated 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 antibodies.

GH-induced signalling and STAT5b nuclear translocation in X-SCID EBV cells transduced with 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. 1.7A, pGC2R γ cells expressed γ c at a normal extent. These cells proliferated in a comparable fashion to control cells following GH stimulation (Fig. 1.7B). Moreover, in WB experiments using an anti-phospho STAT5 antibody, a phosphorylation of the molecule was observed in pGC2R γ cells (Fig. 1.7C). Finally, in reconstituted cells, GH stimulation induced a normal nuclear translocation of STAT5b, as shown in Fig. 1.7D.

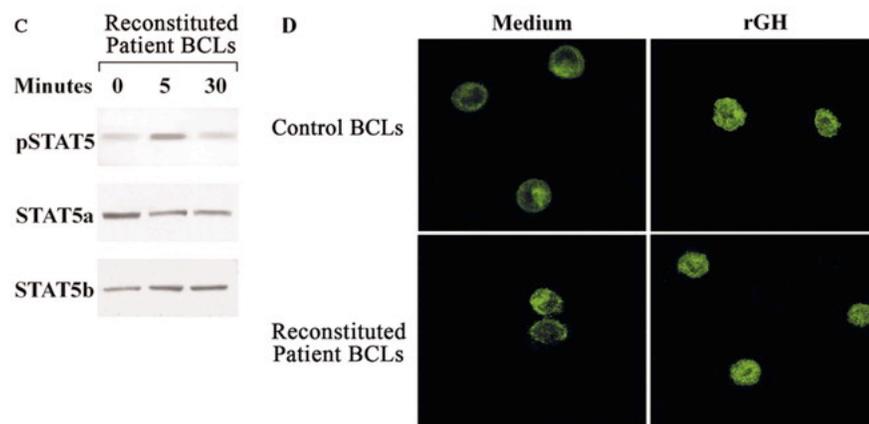
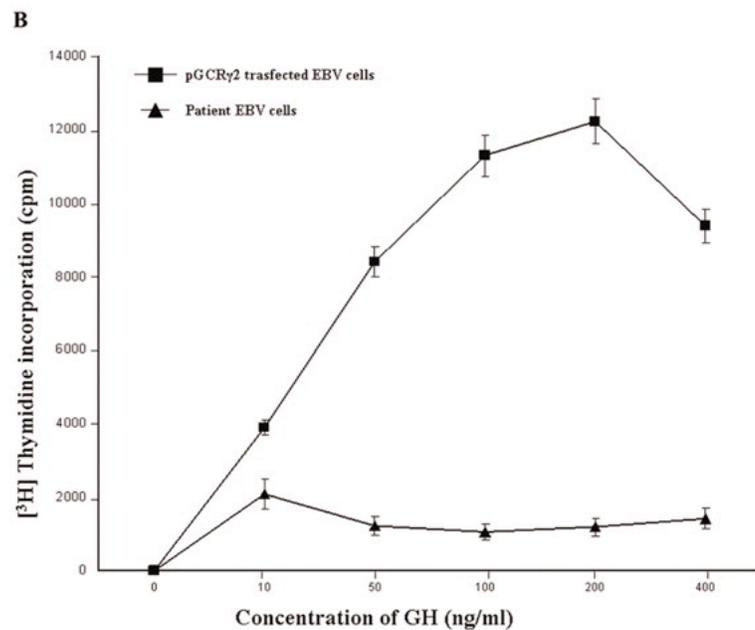
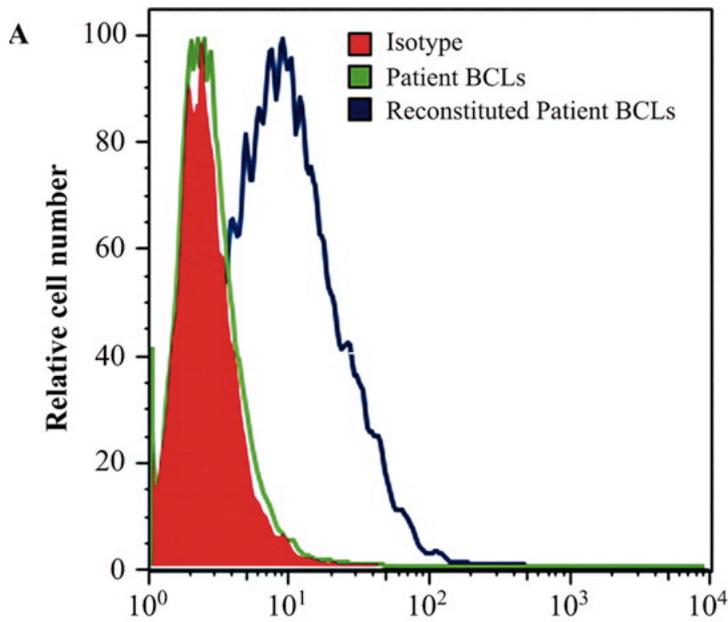


Figure 1.7. GHR signaling in patient BCLs reconstituted with WT γ c (pGC2R γ cells).

(A) Membrane expression of γ c in patient or pGC2R γ 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 [³H]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 pGC2R γ cells. Membranes were incubated, as indicated, with anti-pSTAT5, anti-STAT5a or anti-STAT5b antibodies.

(D) STAT5b subcellular localization through confocal microscopy analysis in control or pGC2R γ cells unstimulated or stimulated with 500 ng/ml rGH for 30 min at 37°C.

Discussion

In the present study, we examined a potential role of the common cytokine receptor γ chain (γ c) 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 γ c negative patients BCLs 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 analysing the pattern of protein tyrosine phosphorylation. In contrast to what 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 γ c negative patients cell lines in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with WT 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 signalling.

STAT-dependent pathways are generally believed to be utilized in cellular events such as cell proliferation, differentiation and apoptosis (55, 56), 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 (57-62), even though STAT5 seems to play a prominent role in receptor signaling. Rodent models of STAT knockouts (63) and the

recent identification of a patient with a homozygous missense mutation of STAT5b gene indicate that STAT5b is essential for a normal postnatal linear growth (64). 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-2R α 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 (65), other signaling pathways also contribute to a full GHR response. GH has been shown to activate the phosphatidylinositol-3 kinase- (PI3 kinase)-protein kinase B signaling (66), mitogen-activated protein kinases and extracellular regulated kinases 1 and 2 (ERK1 and 2) (67-69). 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 PI3 kinase in GH-induced proliferation of BCLs was ruled out since 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 PI3 kinase pathway (70). 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 (71). This could lead to hypothesize differential functions of an individual receptor exerted in different tissues. A cell type-restricted STATs activation has been reported (72-74). STAT5 is not activated following GH stimulation in human

fibrosarcoma cells even though these cells express the STAT5 protein (74), 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 (42, 75). Recently, it has been proposed that STATs tyrosine phosphorylation and nuclear translocation are two events that are regulated separately (54). In particular, Giron-Michel J. and coworkers demonstrated in the hybrid receptor γ_c /GM-CSFR β that γ_c /JAK3 complex controls the nuclear translocation of pSTAT5 rather than STAT5 phosphorylation itself (76). 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 observed in patient cells. Moreover, through confocal microscopy studies, we demonstrated that GHR and γ_c co-localize, as expected in that both molecules are Type I hematopoietin 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. Crosstalk 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 hematopoietic cell differentiation and functioning remains to be elucidated. To note, in CD34+ progenitors, γ_c participates to hematopoietic cell differentiation by interacting with GM-CSFR β . This interaction does not occur in normal natural killer cells or non hematopoietic cells (76). 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 leads to explain what our group previously reported on an atypical X-SCID phenotype and severe short stature associated with growth hormone hypo-responsiveness and abnormal GHR induced protein tyrosine phosphorylation (19), and indicates that growth failure in X-SCID is directly related to the genetic alteration.

CHAPTER 2

ROLE OF THE FOXP1 TRANSCRIPTION FACTOR IN THE DEVELOPMENT AND DIFFERENTIATION OF THE IMMUNE SYSTEM, NAIL AND CENTRAL NERVOUS SYSTEM.

Introduction

The thymus is a complex organ with a central role in the immune System, as it is crucially required for T-cell differentiation and repertoire selection. The thymus consists of numerous lobules, each clearly divided in two main regions on histological ground an outer cortical region, the thymic cortex, and an inner medulla (77). Each of these regions provides the unique combination of cellular interactions, cytokines and chemokines necessary to induce a particular aspect of thymocyte development (77-79). As thymocytes proliferate and mature into the T-cells, they pass through a series of distinct phase marked by changes in the status of T-cell receptor genes, and in the expression of the T-cell receptor, the co-receptors CD4 and CD8 and other cell-surface marker that reflect the state of functional maturation of the cells. Thymocytes at different developmental stages are localized in distinct regions of the thymus. Most of T-cell development takes place in the cortex whereas only mature T-cells are seen in the medulla (80, 81).

Developing thymocytes undergo extensive selection to ensure that mature T cells exported from the thymus are functional and self-tolerant. These processes are dependent on the interaction between thymocytes and thymic stroma and are termed positive and negative selection (82-86). Positive selection is a consequence of whether the TCR on the developing lymphocyte is able to recognize self-peptide within the context of the self-MHC (87, 88). Cells that cannot recognize the MHC:self-peptide

complex die by neglect. In contrast, thymocytes with too high affinity for self-MHC peptide are deleted by negative selection, an active apoptotic process need to remove self-reactive clones (89). The remaining thymocytes that respond with low affinity are selected to develop into CD4⁺ or CD8⁺ single-positive cells (90-92). Positive selection has been associated mainly with cortical thymic epithelial cells (cTEC) and their associated MHC. In contrast, negative selection of thymocytes has been reported to occur either in the cortico-medullary junction (CMJ) or in the thymic medulla (93, 94). A particular intriguing role for the medullary thymic epithelial cells (mTEC) in the induction of central tolerance recently emerged (95-97). Organ-specific proteins are expressed in 1-5% of mTEC implicating a role in tolerance induction to a variety of tissues by the mTEC (84, 85, 97-99). How such a rare population of cells could bring about large-scale tolerance is still to clarify. A role of mTEC cells in the maturation of T regulatory cells was hypothesized (98). Those thymocytes surviving selection mature in the medulla for an average of 14 days before thymic export towards the post-capillary venules at the CMJ (100). The nature of the medullary maturation process is unknown.

In the mouse, thymus anlagen arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut (101, 102) and is colonized by hematopoietic progenitors in midgestation around embryonic day 11.5 (E11.5) (103). At E11.5 the primordial thymic epithelial cells are yet incompetent to fully support T-cell development (103, 104). In both human and mouse this essential capacity is dependent on the transcriptional activation of the *FOXN1* gene in the thymic epithelium.

FOXN1 gene, mapped on chromosome 17 in human and on chromosome 11 in mice, is composed of nine exons, of which exon 1 is non-coding (105). Human and mouse *FOXN1* genes show identical genomic structures, with the same number, location, phase and size of introns (105). The genes located next to *FOXN1* gene are also identical in both species. *FOXN1* has two alternative first exons that show tissue specificity and suggesting the existence of two different promoter sequences (105).

FOXN1 gene encodes for a member of the forkhead/winged helix class proteins, a large family of transcriptional factors that have been implicated in a variety of cellular processes (106, 107). Thus far, more than 100 members of this family have been identified. The growth of the FOX family paralleled the increase in the overall knowledge on the importance of these molecules in the regulation of developmental processes. 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*) (108).

Recessive mutations in the *FOXN1* coding gene are associated with the mice “Nude” phenotype described for the first time by SP Flanagan (109-111). In the 1966, Flanagan published his observation on a new “hairless” phenotype of mice lacking of fur development and with agenesis of the thymus associated with severe immunodeficiency (109, 112). Today, the Nude mice are a widely use model in immunology, dermatology, cosmetic, oncological and transplantation research because of their defect in allo- or xeno-transplantation rejection due to the absence of a functional immune system (113, 114). Since the time of the first description of the Nude mice phenotype, more than 30000 original papers in peer-reviewed journals have been published, and the number of publication using this model is growing year by year, confirming that this model represents a source of precious information for researchers (16). Recently, we described a novel form of SCID caused by an intrinsic defect of the thymus (MIM 601705) in 2 patients born to consanguineous parents originating from a small community in southern Italy. The hallmark of this human novel clinical phenotype is the congenital alopecia and a profound T-cell defect (17). This disease represents the human equivalent of the Nude/SCID murine phenotype (17). In humans as in mice and rats (115), the Nude/SCID phenotype results from alterations of the *FOXN1* gene (known in rats as *whn*) (17, 18). The first described human *FOXN1* mutation was a C792T transition in exon 5 resulting in the nonsense mutation R255X (18). This mutation lies upstream of

the DNA binding and transactivation domain of this transcription factor, so that translated protein, if any, would be completely non-functional, similar to the previously described rat and mouse *Foxn1* mutations (116-118). To date, the first two patients investigated, carrying the R255X mutation in the homozygous state, were born to consanguineous parents originating from a small community in southern Italy (17). Furthermore, from the community records 4 additional patients of previous generations were affected with congenital alopecia and died in early childhood because of severe infections. Due to the geographical location of the village, lying in the mountains between Naples and Salerno, and the elevated rate of endogamy, the population may be considered isolated.

In our study, based on the presence of several cases in the same community, a genetic screening for the presence of *FOXN1* R255X mutation was performed in the attempt to identify an ancestral founder effect. Moreover, we genotyped two microsatellite markers, D17S2187 and D17S1880, which flank the *FOXN1* gene on chromosome 17 to define whether a founder chromosome is shared by subjects carrying the R255X mutation. Since *FOXN1* seems to play a role in the regulation of the differentiation process of both cutaneous and thymic epithelial tissues (119, 120), an accurate physical examination of all subjects carrier for the identified *FOXN1* mutation was also conducted in order to define whether the mutation in heterozygous state was associated with mild clinical signs.

Materials and Methods

Sampling and participants

All the living individuals still resident in the Acerno village and related to the probands' family were interviewed. Information obtained from this survey was verified and integrated using the local church and community records dating back to 1800. Subsequently, all data were recorded and analyzed using the Cyrillic 3 software (Cherwell Scientific 2000, www.cyrillicsoftware.com V3.0.400). After written informed consent, and upon approval of Institutional Ethical Committee, whole blood samples were collected from 843 subjects of the Acerno community, corresponding to approximately 30% of the population, and processed anonymously. Samples were coded by a third party to allow re-testing of heterozygotes to confirm the result of the first determination.

Mutation screening and sequence analysis

Genomic DNA was extracted by salt precipitation method (121) from peripheral blood of the study subjects. 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' (18). PCR amplification resulted in a 184 bp product, containing 7 bp of intron 4, 131 bp of exon 5, and 46 bp of intron 5. PCR was carried out according to the following protocol: 95°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute, and an extension step was performed at 72°C for 7 minutes in a Stratagene® RoboCycler® Gradient 96 thermal cycler (Stratagene®). PCR products were visualized after electrophoresis on a 2% agarose gel and purified in a first step using the High Pure PCR

product purification kit (Boehringer Mannheim). Aliquots 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). A second blood sample was drawn from subjects with the R255X mutation at the heterozygous state to confirm the result of the first determination.

Clinical evaluation

Physical examination of the identified heterozygous subjects was conducted paying a special attention to ectodermal abnormalities and, in particular, to alterations of hair and nails. These annexa were analyzed by at least 2 of us in the all the identified heterozygote carrier subjects, in 23 non heterozygous otherwise healthy married-in subjects, and 50 non heterozygous subjects belonging to the extended pedigree. An accurate anamnesis was performed for each individual to evaluate any systemic disease able to induce nail dystrophy. Whenever necessary, cultures were performed to rule out mycotic infections. Overt psoriasis was also excluded.

Our pathologist performed fetus physical examination after written informed consent from the family and upon approval of Institutional Ethical Committee

Marker typing

Haplotype analysis was performed on 47 chromosomes carrying the mutation. According to the draft human genome sequence at UCSC (<http://genome.cse.ucsc.edu/>; December 2001 freeze), we chose microsatellite markers D17S2187 (GATA70H05) and D17S1880, located at 3.4 and 4.1 Mb centromeric and telomeric from the *FOXNI* gene, respectively. PCR primers for the microsatellite markers were obtained from the

Genome Database (<http://gdbwww.gdb.org>) and the Cooperative Human Linkage Center (<http://gai.nci.nih.gov/CHLC>). PCR conditions were as described above. PCR amplification products were resolved on 6% non-denaturing polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

Results

Identification of a founder effect for the R255X mutation described in the 2 patients with Nude/SCID immunodeficiency.

Fifty-five subjects, corresponding to 6.52% of the studied population, were found to be heterozygous carriers of the mutation. Twenty-eight of them, corresponding to 50.9%, were female. These 55 subjects originated from 39 families, which were subjected to a genealogical study. Through the archival database, common ancestors were identified for the 55 heterozygous subjects. They are linked in an extended 7-generations-pedigree comprising 483 subjects (Fig. 2.1), founded by a single ancestral couple born at the beginning of the 19th century. In one family, heterozygotes were born to a mother who belonged to the pedigree but was found to be genotypically normal in two independent determinations. The family name of the deceased father, who presumably transmitted the mutation, was one of the 6 recurrent surnames in the pedigree of the probands.

From the founding ancestral couple in the first generation, 4 family groups originated. All the affected cases belonged to this pedigree. On the basis of our pedigree analysis, 33.3% of the mutation carriers inherited the mutated gene from their mother, whereas 66.7% inherited the mutant allele from their father. The pedigree also shows a high rate of past and present consanguineous and endogamous matings, typical of small communities. In particular, 14 out of 151 marriages were found to be consanguineous.

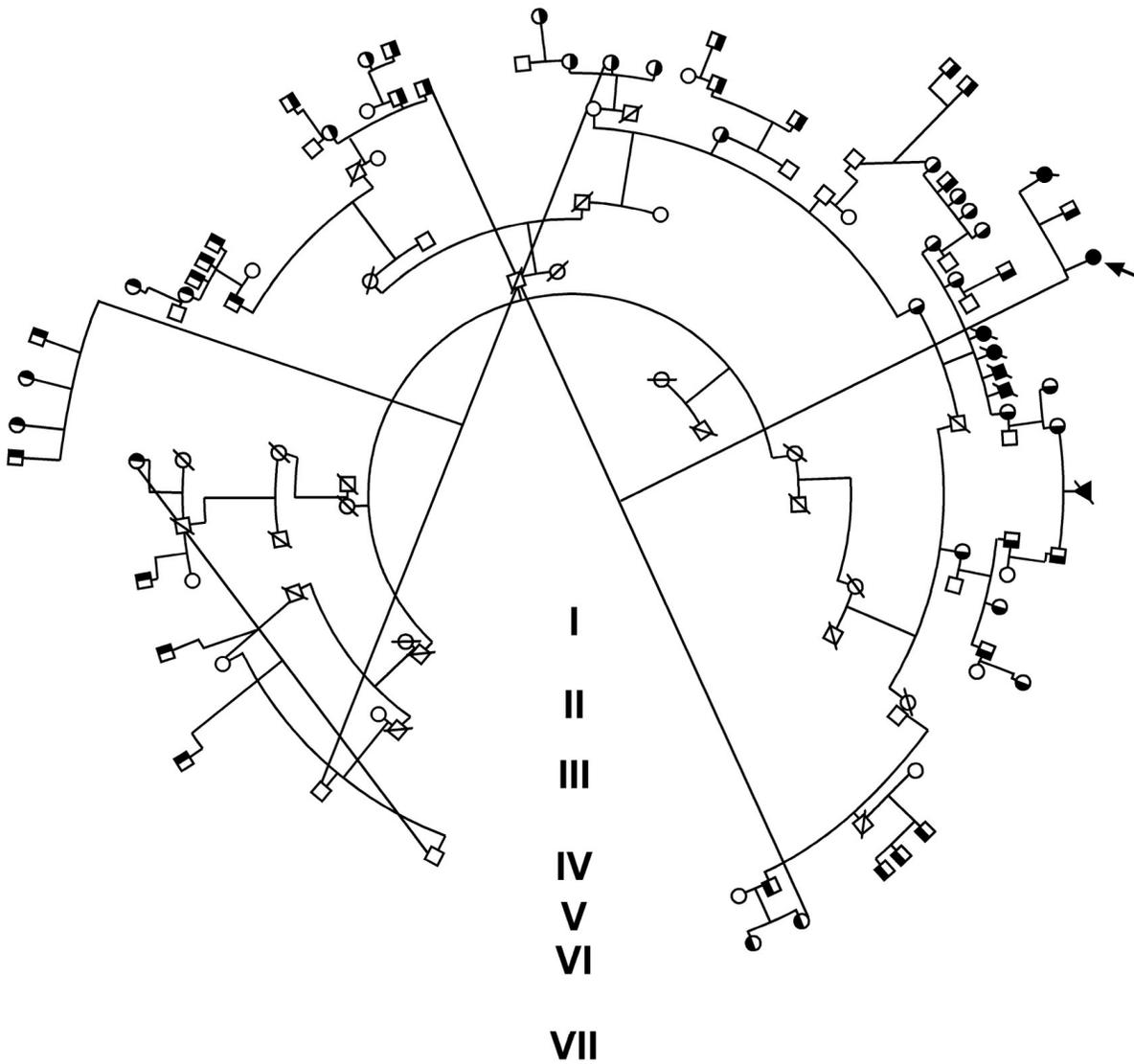


Figure 2.1 Seven generations pedigree including the Acerno families linked to the index family. Not all relatives are shown, but only the subjects necessary to demonstrate the founder effect. Filled circles indicate affected individuals. Half-filled symbols indicate heterozygous carriers of the mutation R255X. The arrow indicates the proband.

To confirm that the R255X mutation represents a single ancestral event, we constructed the haplotype for the *FOXNI* locus for 47 chromosomes carrying the mutation R255X. The haplotype for D17S2187-*FOXNI*-D17S1880 could be established for 27 carrier chromosomes and three different haplotypes were identified: 3-R255X-3 (14/27), 3-R255X-2 (11/27) and 3-R255X-1 (2/27) (Table 2.I). The same haplotypes for the microsatellite markers (D17S2187-D17S1880) were identified in just 1 (3-3), 5 (3-2) and 6 (3-1) non-carrier chromosomes. Among the carrier individuals for whom the phase could not be established (20/47), 3 and 2 were consistent with haplotypes 3-3 and 3-1, respectively, whereas 10, 2 and 3 were consistent with two of the previous haplotypes, 3-3/2, 3-3/1 and 3-2/1, respectively. Assuming recombination over the generations, these haplotypes could be consistent with a single ancestral origin of mutation R255X in the Acerno population.

Table 2.I. Haplotypes analysis		
	R255X carrier chromosomes	
Haplotype	Known fase	Unknown fase
3-R255X-3	14/27	3/20
3-R255X-2	11/27	-
3-R255X-1	2/27	2/20
3-R255X-3/2	-	10/20
3-R255X-3/1	-	2/20
3-R255X-2/1	-	3/20

Identification of clinical signs associated with heterozygous mutations in the FOXNI gene

It is well known that loss of function mutations of *FOXN1* gene are characterized in the both human and mouse Nude/SCID phenotype by the abnormal morphogenesis of epidermis, hair follicles and thymus (119).

Since this, the physical examination of the R255X heterozygous subjects was performed paying a special attention to ectodermal abnormalities and, in particular, to alterations of hair and nails. No gross alteration of the hair was noted in association with the heterozygosity. Differently, nail examination revealed that 39 out of the 55 heterozygous subjects showed a nail dystrophy. This finding was not observed in any of 23 not heterozygous otherwise healthy married-in subjects, and 50 not heterozygous subjects belonging to the same extended pedigree examined. Accurate anamnesis and cultures were performed to exclude any possible acquired form of nail dystrophy and to rule out mycotic infections. Overt psoriasis was also excluded.

The identified pattern of the phenotypic alteration of the nails was variable (Fig. 2.2). The koilonichia or "spoon nail" characterized by a concave surface and raised edges of the nail plate associated with a significant thinning of the plate itself were the more frequent alteration. Koilonichia was found in 28 subjects, whereas the canaliform dystrophy and a transverse groove of nail plate (Beau's line) were found in 13 and 11 subjects, respectively (Table 2.II). It is note worthy that the more specific phenotypic alteration was the leukonychia characterized by a typical arciform pattern, involving the proximal part of the nail plate reminiscent of the half-moon. This is not surprising since it reflects an abnormal differentiation process of the cells of the matrix. Seventeen out of the 39 heterozygotes with nail dystrophy had 2 or more features. It should be noted that in 18 cases fingernails and toenails were both affected, whereas in 4 subjects the dystrophy was only observed in the feet.

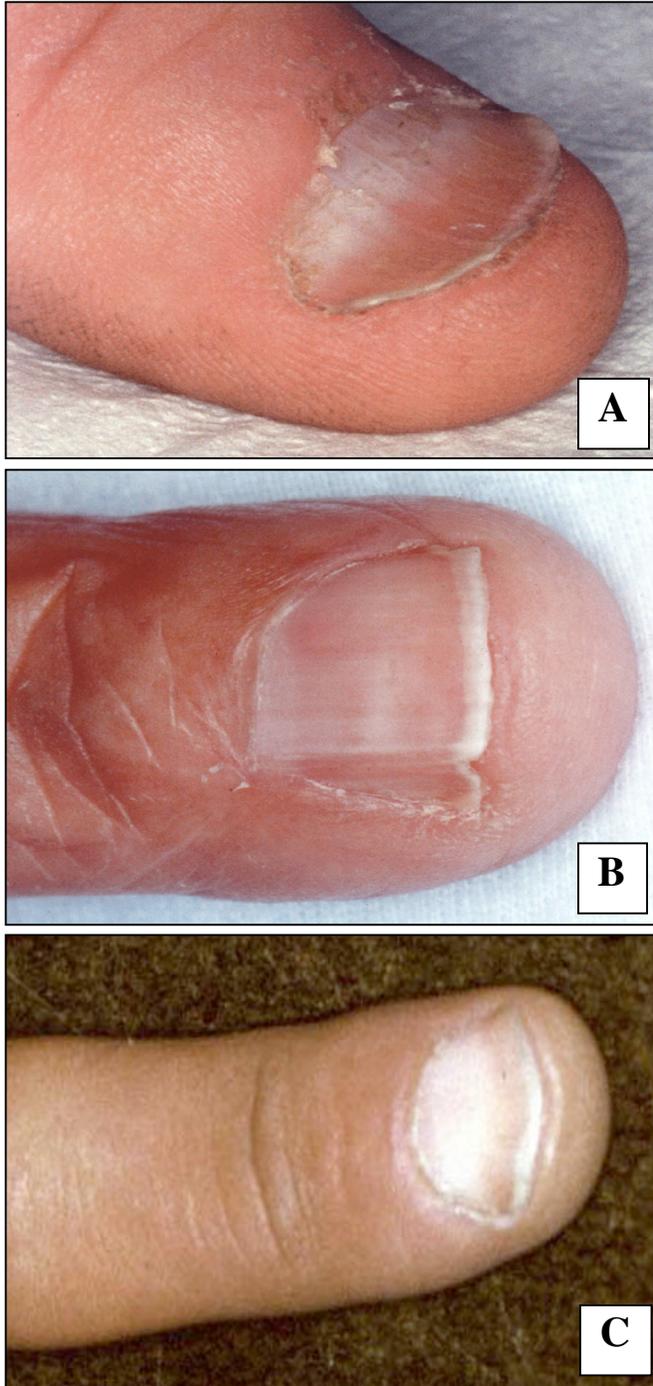


Figure 2.2. Nail dystrophy pattern in subjects heterozygous for the *FOXN1* mutation. (A) Koilonychia. (B) Canaliform dystrophy. (C) Leukonychia.

Table 2.II. Pattern of nail dystrophy in R255X heterozygous subjects

Pattern of Nail dystrophy	N° of Patients	Nail alteration localization	
		Hand	Feet
Leukonychia	6	5	3
Koilonychia	28	23	18
Canaliform Onychodystrophy	13	12	2
Beau's line	11	10	6

Unexpected phenotype of a human Nude/SCID fetus homozygous for the nonsense R255X mutation in exon 5 of the FOXN1 coding gene

During the population study an affected female fetus homozygous for the R255X mutation was identified, thus leading to a medical interruption of pregnancy. 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.

The pathology examination revealed that, identical to the Nude/SCID phenotype of mice, the affected human fetus lacked a thymus (Figure 2.3A), confirming that *FOXN1* is essential for thymic development also in humans. As previously reported in mice model (109, 119), the skin appeared grossly abnormal, being more tight than usual. The histology of the skin revealed 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 (Figure 2.3B, C). While there have been

no prior studies of FOXN1 activity in the nervous system, we observed that the *Foxn1* gene is expressed in murine epithelial cells of the developing choroid plexus, a structure filling the lateral, third, and fourth ventricles of the embryonic brain (Figure 2.4).

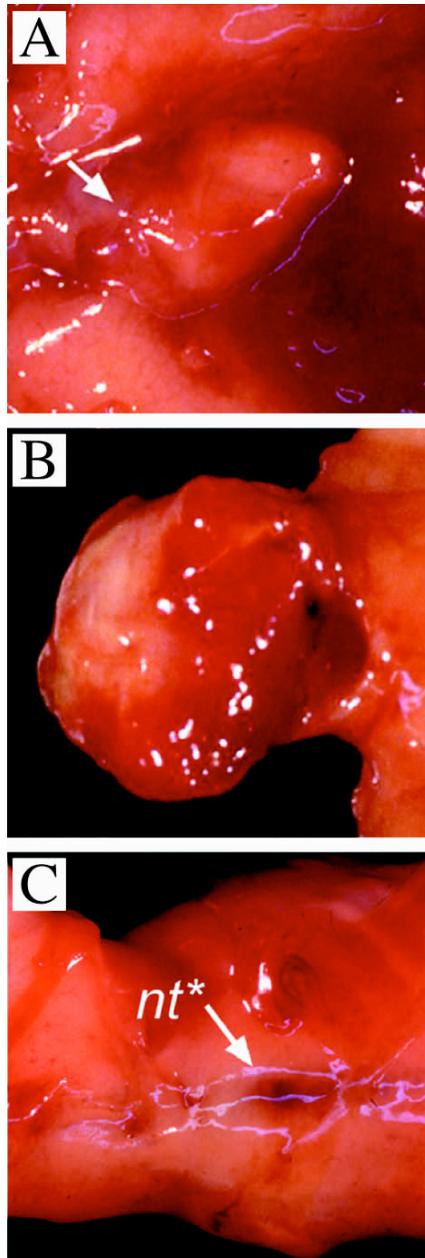


Figure 2.3. Morphological phenotype of FOXN1^{-/-} human. The skin was tight, shiny and smooth. (A) Ventral view of the fetus showing the absence of thymus in the chest. The arrow indicates the usual thymus location. (B) Dorsal view showing the skull of the FOXN1^{-/-} fetus. Complete anencephaly characterized by the absence of scalp, calvarium and brain. (C) Dorsal view showing the craniospinal rachischisis. nt*, open neural tube (spina bifida).

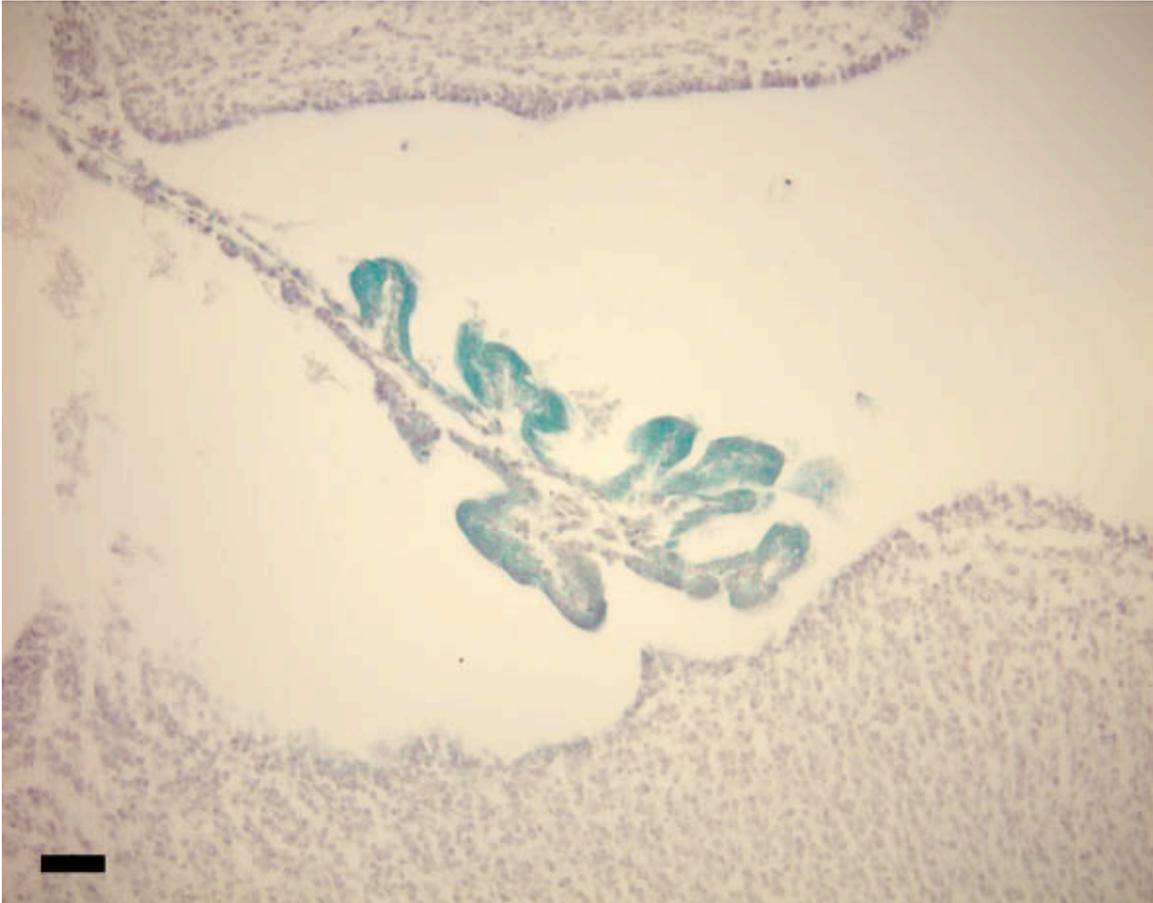


Figure 2.4. *Foxn1* expression in the brain of E15.5 murine embryos. A β -galactosidase reporter gene was inserted into the *Foxn1* locus by homologous recombination (110). Heterozygous embryos (*Foxn1*⁺/*Foxn1*^{lacZ}), which are phenotypically normal, were stained for β -galactosidase activity (blue) as described (119). *Foxn1* is expressed throughout the epithelium of the developing choroid plexus. (scale bar represents 50 μ m). (Figure provided us by our collaborator Doc Brissette J.L.)

Discussion

Reported data demonstrate that the *FOXNI* mutation R255X, which underlies the human equivalent of the murine Nude/SCID phenotype, is present in the heterozygous state in the Acerno population, and seems to have a single ancestral origin, dated to the early 19th century. An extended pedigree, including most of the heterozygous subjects in the population, was constructed on the basis of genealogical data. The prevalence of the carriers in this community was of 6.52% thus leading to a presumptive estimate of expected new cases, in the absence of prenatal diagnosis, of 1:1000 live births. This estimation does not take into account the elevated frequency of the carrier status within the extended pedigree, which corresponds to 36.9%, and, more importantly, the elevated rate of consanguineous matings among the families, which have occurred on several occasions in the last century.

Identification of a large pedigree in an isolated population is suggestive of a common allelic variant that are likely to have the same ancestral origin. Indeed, analysis of microsatellite markers D17S2187 and D17S1880 identified three haplotypes, 3/R255X/3, 3/R255X/2 and 3/R255X/1. Assuming recombination over the generations, these haplotypes could be consistent with a single ancestral origin of mutation R255X in the Acerno population. Our findings further support that all carrier chromosomes derive from a single ancestral chromosome on which the mutation arose in the conserved haplotype.

Nude/SCID immunodeficiency is particularly devastating due to the absence of the thymus, and because stem cell based therapies cannot provide a cure, as they can in the other forms of SCIDs. Thus, the importance of genetic counseling of couples at risk and of first trimester prenatal diagnosis by direct genetic analysis, given the high frequency of *FOXNI* mutation in the Acerno population cannot be overemphasized. It is remarkable that although a screening program for prenatal diagnosis has only been

introduced for a short period of time in Acerno, an affected female fetus could already be identified, thus indicating the usefulness of this effort in an isolated community.

Perhaps, in the near future innovative therapeutic strategies as thymic transplantation, already successfully performed in DiGeorge syndrome, could be proven helpful in treating this form of SCID in affected individuals.

Clinical examination of subjects related to the 2 identified Nude/SCID patients shows that nail dystrophy is frequently associated with heterozygous mutations in the *FOXN1* gene. This finding is in keeping with the alterations of digits and nails reported in a few strains of nude mice (119). Since the nail plate originates mainly from the nail matrix and *FOXN1* is selectively expressed in such structure, our observation confirms that this transcription factor is involved in the maturation process of nails, and suggests that this sign may be indicative of heterozygosity for this molecular alteration. Our observations were independently confirmed in the mouse model by L Mecklenburg and colleagues, which demonstrated the association between the nude mice severe onychodystrophy and alterations of the onycholemmal differentiation pattern (122).

As previously reported, this form of SCID is particularly severe due to the absence of the thymus. Thus, the importance of genetic counseling of couples at risk and of first trimester prenatal diagnosis by direct genetic analysis, given the high frequency of *FOXN1* mutation in the Acerno population cannot be overemphasized. It is remarkable that although a screening program for prenatal diagnosis has only been introduced for a short period of time in Acerno, an affected female fetus could already be identified, thus indicating the usefulness of this effort in an isolated community.

The pathological examination of the homozygous *FOXN1* mutated fetus suggests that *FOXN1* may be involved in the brain and neural tube development and function in a similar fashion to other members of the forkhead/winged helix class proteins, such as murine *HNF-3 β* and *BF-1* (123), and human *FOXP2* (124). Mice defective in *HNF-3 β* through gene targeting technology lack notochord which results in severe defects of the

neural tube organization (123). Similarly in humans, alterations in the *FOXP2* gene were associated with neurodevelopmental disorders, suggesting that the molecule has a key role in neural functioning (124).

Furthermore, as the choroid plexus modulates the composition of the cerebrospinal fluid, transporting or secreting key molecules into the brain's environment, it is thought that plexal cells have profound effects on the morphogenesis of neural structures (125). Thus, the expression of *Foxn1* in the embryonic choroid plexus is consistent with a possible role of *Foxn1* in the development of the central nervous system.

Moreover, 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 during the first months of postnatal life, when the protection of the newborn by the mother immune system declines. 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, since the first trimester mortality is not justified by the SCID *per se*. Furthermore, even though there is not any demonstration that in mice there are grossly brain malformations, it has been reported in Nude/SCID mice a reduced number of Purkinje cells of the cerebellum, which in addition is reduced in size (126). Furthermore, a high prevalence of perinatal death was reported in the spontaneous strain "nude Yurlovo" (127). It is to note that organogenesis in mice lasts all the pregnancy, while in humans it mainly occurs in the first 3 months.

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.

The association herein reported in man, indicating that the human phenotype is more severe than in mice, could also explain the reason by which the human counterpart of the mouse Nude/SCID, described in 1966, has only been identified 30 years later (17). Further studies may be planned to define whether a more severe clinical expression of the Nude/SCID may lead to prenatal lethality and a milder one to a newborn with Nude/SCID.

CHAPTER 3

INTERLEUKIN-12 RECEPTOR BETA 2 CHAIN GENE (*IL-12RB2*) SINGLE NUCLEOTIDE POLYMORPHISMS AND ELEVATED SERUM IGE LEVELS IN CHILDREN.

Introduction

Interleukin-12 (IL-12) is a proinflammatory cytokine that plays a critical role in cellular mediated immunity against intracellular pathogens, such as *Mycobacteria* and *Salmonella* (128).

The inflammatory response triggered by pathogens is characterized by recruitment of inflammatory cells, alteration in vascular permeability and production of soluble mediators, such cytokine, chemokine and interferons (129). The first response of the immune system, innate or specific, is induced by binding of bacterial an microbial ligands to surface receptors expressed on macrophages and dendritic cells and evolved to recognize and bind common constituents of many bacterial surface. Bacterial molecules binding to these receptors trigger the cells to engulf the bacterium and also induce secretion of biologically active molecules by macrophages and dendritic cells (129). The development of an effective innate response to pathogen infections is necessary to prevent or control infections, but is ultimately instrumental for the consequential adaptive immune response, required to complete elimination of pathogens and establishing of memory. The soluble mediators released during the innate response are then critical to regulate and drive the adaptive immune response, mainly guiding the migration of antigen-specific T lymphocytes to lympho nodes, where they interact with antigen-presenting cells (APC) (129). This will results in activation and expansion of antigen-specific T lymphocytes, resulting in a response of T helper 1 (Th1) or T helper 2

(Th2) type, depending on the intracellular or extra-cellular nature of the pathogen, respectively (130). Different pathogens will induce the production of distinct patterns of cytokines and mediators, finally driving T cells towards either Th1 or Th2 phenotype, which are characterized by the ability to secrete different cytokines, resulting in an adaptive immune response cell or antibodies mediated, respectively (130). The main cytokine involved in directing T cells towards Th1 phenotype has been demonstrated to be IL-12 (131, 132), while IL-4 drives Th2 phenotype development (133).

IL-12 is a heterodimeric cytokine composed of a 40 kDa (p40) and 35 kDa (p35) subunits secreted by mature dendritic cells, neutrophils and macrophages (134-136). The p40 subunit is homologous to other members of the cytokine-receptor family, while p35 is homologous to other single chain cytokine, such as IL-6 and G-CSF (137). IL-12 mediates its biological activities through a specific surface receptor (IL-12R) consisting of two polypeptide chains, $\beta 1$ and $\beta 2$. Both subunits belong to the class I cytokine receptor superfamily and are homologous to cytokine receptor glycoprotein (gp)130 (138-140). IL-12 p40 subunit interacts mainly with IL-12R $\beta 1$ chain, while p35 binds to IL-12R $\beta 2$ chain. In the human system, IL-12R $\beta 1$ and $\beta 2$ chains alone have low affinity to IL-12 and they both are required to form high-affinity IL-12 binding sites (141). In contrast in mouse system, IL-12R $\beta 1$ is primarily responsible for the high-affinity binding of IL-12. In both systems, IL-12R $\beta 2$ chain is responsible for signal transduction: indeed it contains 3 tyrosine residues, whose phosphorylation activates the intracellular signaling cascade (142).

IL-12R is mainly expressed on the surface of activated T and NK cells (142), but it is also found on DC's (143) and B cells (144). IL-12 expressed at low levels in resting T cells is up-regulated following activation of T cells through TCR, and further increased by IL-12 itself, IFN- α , IFN- γ , TNF and CD-28 co-stimulation (129). The expression of IL-12R $\beta 2$ is highly regulated and is crucial in conferring responsiveness to IL-12. Only Th1 cells retain IL-12R $\beta 2$ expression and consequently can respond to IL-

12, while Th2 cells down-regulate IL-12R β 2 losing the ability to respond to IL-12 (145). In Th1 cells, the IL-12R β 2 expression is mainly maintained by IFN- γ -mediated up-regulation of the transcription factor T-bet (146). In contrast, in Th2 cells IL-4 inhibits IL-12R β 2 expression (147).

Upon binding of IL-12 to its receptor, receptor-associated protein tyrosine kinases of the Janus kinases family (JAKs) (32), namely JAK2 and TYK2, are activated by transphosphorylation (148, 149). JAK2 and TYK2 in turn phosphorylated the 3 tyrosine residues on the intracellular portion of IL-12R β 2, creating a docking site for the transcription factors Signal Transducers and Activators of Transcription (STATs). STATs bind to the receptor chain and get in turn phosphorylated by JAKs. Phosphorylated STATs homo- or hetero-dimerize and translocate in the nucleus, where they regulate gene transcription. Although STAT1, STAT3 and STAT5 have been reported to be activated in response to IL-12 (142), the specific functional effects of IL-12 are mainly due to the activation of STAT4 (129).

Even though IL-12 stimulates proliferation of hematopoietic precursor cells and of activated T and NK cells, its principal function is the strong induction of IFN- γ production.

Both IL-12 and IFN- γ suppress IgE synthesis and inhibit the development of Th2 cells (150). Allergic disorders are thought to be associated with a predominant Th2 activity, usually involved in the response to extra-cellular pathogens (151). Furthermore, accumulating evidence indicates that a defective Th1 response is associated with elevated antigen specific IgE levels (152). Mice lacking IL-12 or IL-12R chains exhibit poor Th1 and enhanced Th2 responses (153-155). Mutations of IL-12R beta 2 gene were reported in adult atopic subjects (156, 157) as well as polymorphisms of Fc ϵ RIIB and IL-4R coding genes.

Thus, to verify the possible association between enhanced Th2 response and IL-12R β 2 coding gene (*IL-12RB*) single nucleotide polymorphisms (SNPs) in childhood, a

systematic search for nucleotide variations in the gene coding for this receptor chain was performed in a cohort of atopic patients with elevated serum IgE level and healthy control. Allelic frequency of each identified polymorphism was calculated in the control group and in atopic patients divided in two subgroups according to the serum IgE level. Recently, Ober et al (158) have reported data suggesting that the study of haplotypes at multiple loci in a gene could be more informative than the separate study of separate SNPs. To test the relationship between IgE value and *IL-12RB2* polymorphisms haplotype analysis was also performed.

Materials and Methods

Patients

Patients with elevated serum IgE levels (53 unrelated individuals with a mean age of 8.38 ± 3.34 years; 29 male) met the following inclusion criteria: High serum IgE levels (Total serum IgE > mean value for age + 2SD), absence of typical clinical and immunological features for Hyper-IgE Syndrome (159). The main clinical features of the patients were reported in Table 3.I.

Main clinical features	Number of patients	Number of patients according to serum levels	
		<2000 U/ml	>2000 U/ml
Asthma	27	15/34	12/19
Rhinitis/Conjunctivitis	12	5/34	7/19
Eczema/Atopic Dermatitis	11	6/34	5/19
Urticaria	15	8/34	7/19

Patients were divided on the basis of the IgE levels in 2 subgroups: group A consisted of children with serum IgE levels > 2000 kU/l and group B of children with IgE levels > mean value for age + 2SD < 2000 kU/l. Fifty healthy unrelated control subjects were randomly selected.

The protocol was approved by the local ethics Committee, and informed consent from all individuals was obtained.

Genomic DNA isolation. PCR amplification and exons sequencing

DNA was isolated from peripheral white blood cells using the salting-out method (121). IL-12RB2 gene exons were amplified by 30 cycles of PCR using the specific primers listed in Table 3.II:

Each amplified product was run on a 1.8% agarose gel with a size marker (1 kb DNA ladder M-Medical GENENCO - Life Science). These fragments were purified from gel using Gel extraction kit (QIAquick) and sequenced in an automatic DNA sequencer (ABI 377 DNA sequencer, Applied Biosystems).

Statistical analysis

The association between the IL-12RB2 gene polymorphisms and IgE levels was investigated by comparing each allele rate in patients and controls groups by Pearson χ^2 test.

Table 3.II: IL-12R primers

Name	Sequence (5'- 3')	5'-Position
1PRB2L	5'-tgttgagaagcattgtta-3'	Promotore
2PRB2R	5'-cttcatttgatttgctgt-3'	Promotore
PRB2L5'A	5'-tcacaatccataaagacaa-3'	Promotore
PRB2R3'	5'-aactgccaggtaaacaataa-3'	Promotore
EX1L5'	5'-agacagcaaatacaaatgga-3'	Esome 1
EX1R3'B2	5'-gagacgcgagacacgcaatc-3'	Esome 1
1EX1L5'B2	5'-actccagtggcggtcttct-3'	Esome 1
2EX1R3'B2	5'-cgaggtgaggtgcgtttatg-3'	Esome 1
EX2L5'B2	5'-gctattttggctctggtatt-3'	Esome 2
EX2R3'B2	5'-actacaaaagaccaactacc-3'	Esome 2
EX3L5'B2	5'-ttaattttaccctgttttg-3'	Esome 3
EX3R3'B2	5'-aatgctcaccaccaacgaag-3'	Esome 3
EX4LB2	5'-aggttttgcttttctttt-3'	Esome 4
EX4RB2	5'-aatacagcctctcactcact-3'	Esome 4
EX5LB2	5'-atthttgcttttctactgt-3'	Esome 5
EX5RB2	5'-aatgaaacagtgaataaaa-3'	Esome 5
EX6LB2	5'-tttgtttgctctggtcata-3'	Esome 6
EX6RB2	5'-cttcagtgtttttcctca-3'	Esome 6
EX7LB2	5'-taggtgtgaaaacaa-3'	Esome 7
EX7RB2	5'-gatcaaacaggagtttg-3'	Esome 7
EX8LB2	5'-cctttgaaacagagcctact-3'	Esome 8
EX8RB2	5'-gttttattcccttgagttt-3'	Esome 8
EX9LB2	5'-gtctgggagtaaatagtga-3'	Esome 9
EX9RB2	5'-aagagatgagtaagacacag-3'	Esome 9
EX10LB2	5'-tttgtttctcctctttctg-3'	Esome 10
EX10RB2	5'-tcccaacaatgtgaact-3'	Esome 10
EX11LB2	5'-gaaagcatttgatcagata-3'	Esome 11
EX11RB2	5'-tgtcaacctccaaatccaaa-3'	Esome 11
EX12LB2	5'-cctcctgtgccctactttac-3'	Esome 12
EX12RB2	5'-acctccttggttttctga-3'	Esome 12
EX13LB2	5'tatgctgtggagtgagtgag-3'	Esome 13
EX13RB2	5'cagagtttgagtaggcgtatg-3'	Esome 13
EX14LB2	5'-tcctttctgacctggct-3'	Esome 14
EX14RB2	5'-ccaggaatgtgtgagagc-3'	Esome 14
EX15LB2	5'-ctttgctttcag ttcgat-3'	Esome 15
EX15RB2	5'-tagggggttttagttc-3'	Esome 15
EX16LB2	5'-ctcaggagaagaagacac-3'	Esome 16
EX16RB2	5'-ttgaggggaaaacagaaagg-3'	Esome 16

Results and Discussion

Two newly identified SNPs, one G2569A in the exon 14 and the other A2977C in exon 16 of IL-12RB2 gene were found. G2569A polymorphism maps in the transmembrane region, while the A2977C SNP maps in the Box 2 domain of the beta 2 chain. Both of them did not result in a change in the aminoacid sequence.

Allele frequency	Controls (n = 50) (%)	Patients (n= 53) (%)	OR	95% CI	P
G2569A	27	36	1.52	0.83-2.77	0.1707
A2977C	29	41.9	1.77	0.99-3.19	0.0547

Frequency of G2569A and A2977C SNPs did not differ significantly between patients and controls when the overall group of patients was analyzed ($p = 0.1707$ and $p = 0.0547$, respectively) (Table 3.2).

However, when the patients were divided on the basis of IgE levels as specified in the method section, in the group A a significantly higher frequency of A2977C SNP than controls was observed (odds ratio [OR], 1.85; 95% CI, 1.03-3.32; $p = 0.039$) (Table 3.3).

Table 3.IV. IL-12 SNPs frequency in patients with IgE serum levels .2000 U/ml (group A) and controls

Allele frequency	Controls (n = 50) (%)	Patients (n= 19) (%)	OR	95% CI	P
G2569A	27	36	0.95	0.71-1.34	0.87
A2977C	29	41.9	1.85	1.03-3.32	0.039

Recently, Ober et al (158) reported data suggesting that the analysis of haplotypes at multiple loci in a gene could be more informative than the analysis of a separate SNP. Thus, to test the relationship between IgE levels and IL-12RB2 SNPs, haplotype analysis was also performed. The genotype carrying both alleles in the reference sequence, referred as "wild-type allele", was significantly more expressed in the control subjects than in patients (29.2 vs 7.5%; $p < 0.001$). By contrast, in the patients' group 15.1% was homozygous for the A2977C SNP as compared to the 4.2% of controls ($p < 0.008$).

In conclusion, differently from what previously reported on adult patients, we could not find any mutation of IL-12RB2 gene in atopic children, but two new SNPs, 2569G/A and 2977A/C, mapping in the exon 14 and exon 16, respectively. The frequency of 2977A/C SNP was more represented in atopic children. Since no aminoacid change occurred, this alteration would represent a molecular marker, suggesting a role in atopy of a gene in close linkage disequilibrium with the reported SNPs.

CONCLUSIONS

Human body, with its multitude of complexities, requires harmonious interaction among all its constituents for the maintenance of homeostasis. Because every member of this intricate community has its own “agenda”, a peaceful and productive interaction between them is not trivial.

Communication between and within cells is essential for the development and survival of any complex organism. Homeostasis in the living organism is due to the capacity of several systems to participate in complex networks and to exchange information through cell-to-cell interactions and soluble chemical mediators.

The human Immune System (IS), allows the human body to respond to foreign invaders (bacteria, viruses and other infectious agents) and stressed cells and is the prototype of this type of network. The interactions between specialized cells of the IS and different organs are tightly regulated. Cytokines, hormones and cell-to-cell interactions ensure that the immune cell repertoire is sufficiently devoid of cells that could cause harm to self, while maintaining a wide selection of those that are adept to taking appropriate action against non self. Furthermore, chemical messengers and physical interaction between different cell populations participate in the active regulation of the immune response. These checks and balances are essential to maintain homeostasis.

In the last twenty years of the 20th century, many scientific papers have been published on the molecular and cellular basis of the immune response and on the mechanisms involved in the correct development of immunological diversity and self-tolerance. The product of such studies is formidable. Today, we know the structure of the main molecules involved in the discrimination between self and non-self and we can discern between different cell subpopulations involved in the immune response. Furthermore, we are expanding our knowledge of the genetic and molecular basis of

differentiation and biochemical processes. Less is currently known about the mechanisms involved in the regulation of these processes, about how the cells of the IS and their products interact with the other components of the organism, and about the consequences of these interactions.

During the four years of my PhD program I have contributed to the definition and characterization of these interactions.

Studying a group of atypical patients with X-SCID I could demonstrate for the first time a previously unappreciated functional interaction between the GHR apparatus and the γ c chain. Our study also indicates that growth failure in X-SCID is directly related to the genetic alteration and not only to the immunological status of patients. The identification of unexpected neurological defects in a fetus homozygous for the R255X mutation in the *FOXN1* gene, responsible for the human Nude/SCID phenotype, for the first time suggests that *FOXN1* may be involved in the brain and neural tube development and function. This observation can explain the high rate of abortions in the first trimester in the marriages between subjects heterozygous for the *FOXN1* mutation, whereas other congenital SCIDs are not related to increased spontaneous interruption of pregnancy. Furthermore, the involvement of FOXN1 in the development observed, indicating that the human phenotype is more severe than in mice, could also explain the reason by which the human counterpart of the mouse Nude/SCID, described in 1966, has only been identified 30 years later.

Overall, all my studies underlay the close interaction between different biologic system in a complex regulatory network.

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APPENDIX

SCIENTIFIC PUBLICATIONS DURING THE PHD PROGRAM

- I. Adriani M., Martinez-Mir A., Fusco F., Busiello R., Frank J., Telese S., Matrecano E., Ursini M.V., Christiano A.M., Pignata C.. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. *Ann Hum Genet.* 2004 May;68(Pt 3):265-8.
- II. Salerno M., Busiello R., Esposito V., Cosentini E., Adriani M., Selleri C., Rotoli B., Pignata C.. Allogeneic bone marrow transplantation restores IGF-I production and linear growth in a gamma-SCID patient with abnormal growth hormone receptor signaling. *Bone Marrow Transplant.* 2004 Apr;33(7):773-5.
- III. Busiello R., Adriani M., Locatelli F., Galgani M., Fimiani G., Clementi R., Ursini M.V., Racioppi L., Pignata C.. Atypical features of familial hemophagocytic lymphohistiocytosis. *Blood.* 2004 Jun 15;103(12):4610-2. [Epub 2004 Jan 22.]
- IV. zur Stadt U, Beutel K, Weber B, Kabisch H, Schneppenheim R, Janka G, Busiello R., Galgani M., De Fusco C., Poggi V., Adriani M., Racioppi L., Pignata C. A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype. *Blood* 2004 104: 1909-10.
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