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Congenital Immunodeficiencies: novel diagnostic and therapeutic approach

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BACKGROUND AND AIM

The immune system is a complex integrated network of chemical and cellular mediators that developed during evolution to defend the body from any form of chemical, traumatic or infective insult to their integrity.

A proper immune response relies on the innate immunity, that is responsible for a first line of defense against aggression and the aspecific recognition of a limited repertoire of antigens, and, later, on the adaptative immunity which includes chemical and cellular mediators responsible for a more powerful and specific defensive response from any form of antigen. Alterations of any part of the immune response results in failure of host defense and, in particular, of immunodeficiency, autoimmunity and cancer predisposition.

Recent evidence highlights that the skin participates in a host defenses either acting as a primary boundary for germs, as the principal site of environment–host interactions, or directly in the developmental process of the immune system. As a matter of fact, skin and skin annexa abnormalities, such as skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, can be not infrequently associated with distinct forms of immunodeficiency and may be a warning sign of immunodeficiency, since both epidermal and thymic epithelium have ectodermal origin.

Severe combined immunodeficiency diseases (SCIDs) represent a heterogeneous group of rare genetic syndromes responsible for severe dysfunctions of the immune system, which share similar clinical manifestations. SCID is the most severe form of inherited primary immunodeficiency (PID) and its prevalence is approximately 1:100,000 live births, with a higher prevalence in males (1). SCIDs are difficult to recognize clinically because so many different infectious scenarios

can occur. Without a functional cellular and humoral immune system SCID patients are susceptible to recurrent infections such as severe bacterial, viral, or fungal infections early in life and often present with interstitial lung disease, chronic diarrhea, and failure to thrive. In addition, some patients develop skin rashes, usually caused by maternal T cells transplacental engraftment during fetal life or by a wide autoreaction due to the activation of autologous T cells against skin components (2, 3).

Patients affected with particular forms of PID show anincreased susceptibility to cancer. In particular, a high cancer susceptibility has beenreported for a rare form of PID calledAtaxia Telangiectasia (A-T) whose clinical hallmark is represented by the cerebellar neurodegeneration with the loss of Purkinje cells. Recently, in a few clinical trialsit has been documented that a short-term treatment with glucocorticoids (GCs) is able to partially rescue either the A-T neurological phenotype and lymphocytes proliferation, even though the mechanism of action has not yet been defined (4-7).

Conventionally, SCIDs have been so far classified, on the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical immunological phenotypes related to a specific genetic defect. T cell–deficient but normal B cell (T−B+) SCID and bothT cell– and B cell–deficient (T−B−) SCID, in the presence or absence of NK cells (8). This classification helps in directing molecular studies toward a certain genetic alteration, since it is representative of the stage where the blockage occurs during the differentiation process.

More recently, advances in next generation DNA sequencing allowed new gene identification through whole exome or whole genome sequencing (WES, WGS)

of several forms of PIDs of unknown causes making the genetic identification of immunodeficiency syndromes more efficient (9). Only in the last two years, using this technology 34 new gene defects have been identified.Most of these immunodeficiencies are rare, even though some of them occur more frequently than what previously reported,as documented by several groups (10). Based on the principle of massively parallel sequencing, NGS technology provides an advanced tool to dramatically increase the speed at which DNA can be sequenced at a lower cost as compared to the traditional Sanger sequencing approach.

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

This thesis reports the results obtained during my PhD course in "Human Reproduction, Development and Growth" (XXVIII Cycle) from 2013 to 2016. During these years my research has been focused on the study of the following lines of research:

- role of the autophagy pathway in the pathogenesis of Ataxia Telangiectasiaand the potential positive effect of glucocorticoids on the rescue of a proper cell clearance process in lymphocytes of the patients affected with this disease;
- role of the common gamma chian (γc) in cell cycle progression and survival of continuous primary human malignant cell lines;
- thymic stromal alterations responsible for immunological disorders;
- application of Next Generation Sequencing technique for the diagnosis of immunodeficiencies.

CHAPTER I

Ataxia-Telangiectasia

Ataxia Telangiectasia (A-T) (http://omim.org/entry/208900) is a rare genetic disorder of childhood due to mutations in the Ataxia Telangiectasia Mutated (ATM) gene. To date, over 600 distinct *ATM* mutations have been reported (www.hgmd.cf.ac.uk/ac/gene.php?gene=ATM). Different types of ATM gene mutations are associated to different forms of the disease. Truncating mutations cause the classic form of A-T associated to the complete absence of the protein function. Splice site mutations or missense mutations with expression of the protein at some extent, thus exerting residual kinase activity, are responsible for milder forms of A-T (11).

ATM exerts a central role in the signal-transduction pathway activated by DNA double strand breaks (DSBs), and, therefore, A-T is considered the prototype of the DNA-repair defect syndromes. In response to DSB formation, cell cycle arrest and DNA repair occur by the activation of nuclear form of ATM and several DNA-repair and cell cycle checkpoint proteins (12). Defects in DSBs repaircould also account for the high incidence of chromosomal rearrangements involving the sites of the immune system genes. Of note, gene rearrangements and DSBs repair by ATM are required for a proper immune cell maturation. ATM deficiency may affect VDJ recombination but poorly lymphocyte development. In B cells, the absence of a functional ATM leads to a defect in class switch recombination (CSR), thus supporting a role of ATM in this process (13).

There is also evidence in favor of an extranuclear role for ATM, not involving the DNA damage repair process (14). In ATM-deficient cells, the absence of

cytosolic functional ATM leads to a reduced internalization of phytohaemagglutinin (PHA), depolarization in response to extracellular K^+, defective Ca_2^+ mobilization, a decrease in the duration of Ca_2^+ and Na^+ firing and defective signaling (15). In the cytoplasm, ATM exists as a soluble protein or inside subcellular organelles, such as peroxisomes, important for β-oxidation of fatty acids and detoxification, and endosomes, involved in endocytosis and intracellular routing of receptors and other molecules. Possible targets of cytosolic ATM are the 4EBP1 protein (15), and other molecules involved in oxidative metabolism or in cell protection from the metabolic stress (14).

The clinical phenotype consists of oculocutaneous teleangiectasia, immunodeficiency, high incidence of neoplasms and hypersensitivity to ionizing radiations. The hallmarks of the disease are related to the progressive neurological degeneration, with a selective depletion of Purkinje cells, which greatly impairs the quality of life, invariably confining the patients to wheelchair (16, 17). Together with the neurological degeneration, A-T patients show immunodeficiency, usually affecting both humoral and cellular responses, even though severe infections are not frequent in these patients. Infections in these patients mainly involve the respiratory tract and seem to be related to the neurological alterations themselves rather than to the immune defect (18). The prognosis for survival is poor. To date, A-T remains an incurable disease that leads relentlessly to death around the third decade of life predominantly for progressive neurodegeneration, pulmonary failure with or without identifiable pneumonia, and cancer (12, 19).

1.1 Evaluation of the potential role of autophagy in the pathogenesis of Ataxia Telangiectasia

Ataxia-Telangiectasia clinical hallmark is represented by the cerebellar neurodegeneration with the loss of Purkinje cells (20). Recently, in a few clinical trialsit has been documented that a short-term treatment with glucocorticoids (GCs) is able to partially rescue the A-T neurological phenotype and lymphocytes proliferation even though the mechanism of action has not yet been defined (4-7).

Evidence exists that ATM exerts additional functions in the cytoplasm independent of its role in the DNA damage response (21), such as participation to the autophagy pathway (22). To date, many studies have been performed to identify the pathogenic mechanism responsible for the disease, mainly focusing on the nuclear activity of ATM protein. Thus, the pleiotropic aspects of the phenotype have only partially been clarified.

Autophagy alterations have been implicated in several chronic nervous system disorders, such as proteinopathies (Alzheimer's, Parkinson's, Huntington's diseases) and acute brain injuries (23), whose hallmarks are organelle damage, synaptic dysfunction and neuronal degeneration. Autophagy, known originally as an adaptive response to nutrient deprivation in mitotic cells, including lymphocytes, is now recognized as an arbiter of neuronal survival and homeostasis in that neurons are post-mitotic cells, which require effective protein degradation to prevent accumulation of toxic aggregates. Reactive oxygen species (ROS) generation from dysfunctional mitochondria, as documented in cells from A-T patients, is a potent trigger of autophagy, which acts to clean up damaged organelles (24). It has been demonstrated that in the presence of elevated ROS levels, the activation of the serin/threonine kinase ATM in the cytoplasm leads to activation of LKB1 tumor

suppressor gene, which, in turn, phosphorylates and activates AMP protein kinase (AMPK) (25). AMPK regulates several metabolic processes and activates Tuberous sclerosis complex 2 (TSC2), which participates in energy sensing and growth factor signaling (26). TSC2, by inhibiting the GTPase Rheb, is able to repress mTOR kinase, a key regulator of the protein synthesis and cell growth, thus leading to the activation of autophagy (27). The repression of mTOR complex 1 (mTORC1) signaling results in the absence of phosphorylation of p70 ribosome S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1), direct targets of the mTOR kinase, involved in protein synthesis and survival.

Thus, a direct effect of ATM-mediated inhibition of mTORC1 is the activation of autophagy, a dynamic process, which includes the initiation, formation, maturation and degradation of autophagosomes (APs). The lipidation of LC3 molecule, through the conjugation of phosphatidylethanolamine (PE) converting the cytosolic LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), is essential for autophagy induction, and thus for APs visualization by transmission electronic microscopy (TEM). The lipidated LC3-II form may also be visualized under fluorescence microscope in that it shows a punctate staining pattern and by western blot in that it has faster electrophoretic mobility compared with diffused LC3-I form (28). An increase of LC3-II form may result from either an enhancement of APs biogenesis or inhibition of APs degradation, or may be due by other mechanisms (28). p62/sequestosome (SQSTM1), is a further biomarker widely used to evaluate the appropriateness of autophagic activity. It can bind LC3 protein to promote the recruitment of unwanted material into autophagosomes and the subsequent degradation through autolysosomes. When a normal autophagic flux occurs, p62, as substrate of autophagy itself, is degraded (29). Thus, increased LC3-

II and decreased SQSTM1 levels are indicative of an appropriate autophagic activity whereas increased SQSTM1 levels, reveal defective autophagy (29).

Recently, genes involved in autophagosomal/lysosomal biogenesis and lysosomal functionality, belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network, have been described as crucial for a proper cell clearance through autophagic process (30) during catabolic conditions. In particular, the expression of the *UVRAG* (UV Radiation Resistance Associated) gene plays a pivotal role in the first phase of the process, which consists in the APs biogenesis and formation (31, 32). To ensure a proper degradation of unwanted material and thus cell clearance, the APs must fuse to the lysosomes that contain several active hydrolases, such as β-glucosidase, β-glucuronidaseand cathepsins (30). The fusion process depends on the intracellular positioning of lysosomes in that, only the lysosomes that are transported along microtubules in the perinuclear area, thanks to kinesins (such as KIF2A and KIF1B-β and the monomeric GTPase ARL8B), are able to fuse with APs (33). Moreover, *Vps11* and *Vps18* (Vacuolar Protein Sorting 11 and 18) proteins products are also involved in vesicular trafficking to allow the encounter between APs and lysosomes, resulting in their fusion (34). Eventually, the formation of autolysosomes (ALs), whose content is degraded by lysosomal enzymes and recycled, leads to preserve cellular homeostasis.

In the present work, we evaluated whether abnormal autophagy may be involved in the pathogenesis of A-T by analyzing the autophagic vesicles and the genes implicated in the different stages of autophagy process.

To address the involvement of abnormal autophagic process in the pathogenesis of A-T, we first analyzed, under TEM, the lymphocytes isolated from A-T patients or healthy controls maintained in normal culture conditions or in a

serum-starved condition, which is a classical pro-autophagic stimulus. Ultrastructural analysis of healthy control lymphocytes by TEM showed the morphological aspects of a cell in unstarved culture conditions (Figure 1A) and the accumulation of autophagic vesicles (AVs) at different maturation stages (i.e. autophagosomes and autolysosomes) in a cell after serum starvation for 2h (Figure 1B). Ultrastructural analysis of A-T patient lymphocytes at basal conditions revealed a general increase in AV size and number (Figure 1C-F), not observed in healthy control cells. In the Figure 1D, autophagosomal vesicles with a double-membrane profile in the process of engulfment of targeted organelles in A-T lymphocytes are shown. In the Figure 1E, ALs containing degraded material and, in the Figure 1F, vacuolar structures in the late stage of the autophagolysosomal pathway are shown.

Next we analyzed distinct types of AVs, such as autophagosomes and autolysosomes. TEM revealed that A-T patients exhibit higher number of APs and decrease in ALs, as compared to healthy subjects (mean: 13.60 *vs* 2.30/100 μm^2; $p = 0.03$; mean: 2.10 *vs* 4.7/100 μm^2 respectively) (Figure 1G). This suggests that fusion of APs to lysosomes and, thus, their conversion to autolysosomes, could be impaired in A-T. A further quantitative analysis of ALs revealed that, in the A-T patients, they were not detected in the majority of the fields analyzed, differently from the controls (83 *vs* 36% ALs^- fields, respectively) (Figure 1H), resulting in an APs/ALs ratio much higher in the patients than in the controls (12.22 *vs* 0.93; $p = 0.03$) (Figure 1I). Indeed, the APs number did not further increase under starvation in patient cells, indicating that in A-T an inhibition of autophagy flux rather than a stimulation of APs biogenesis occurs (Figure 1J).

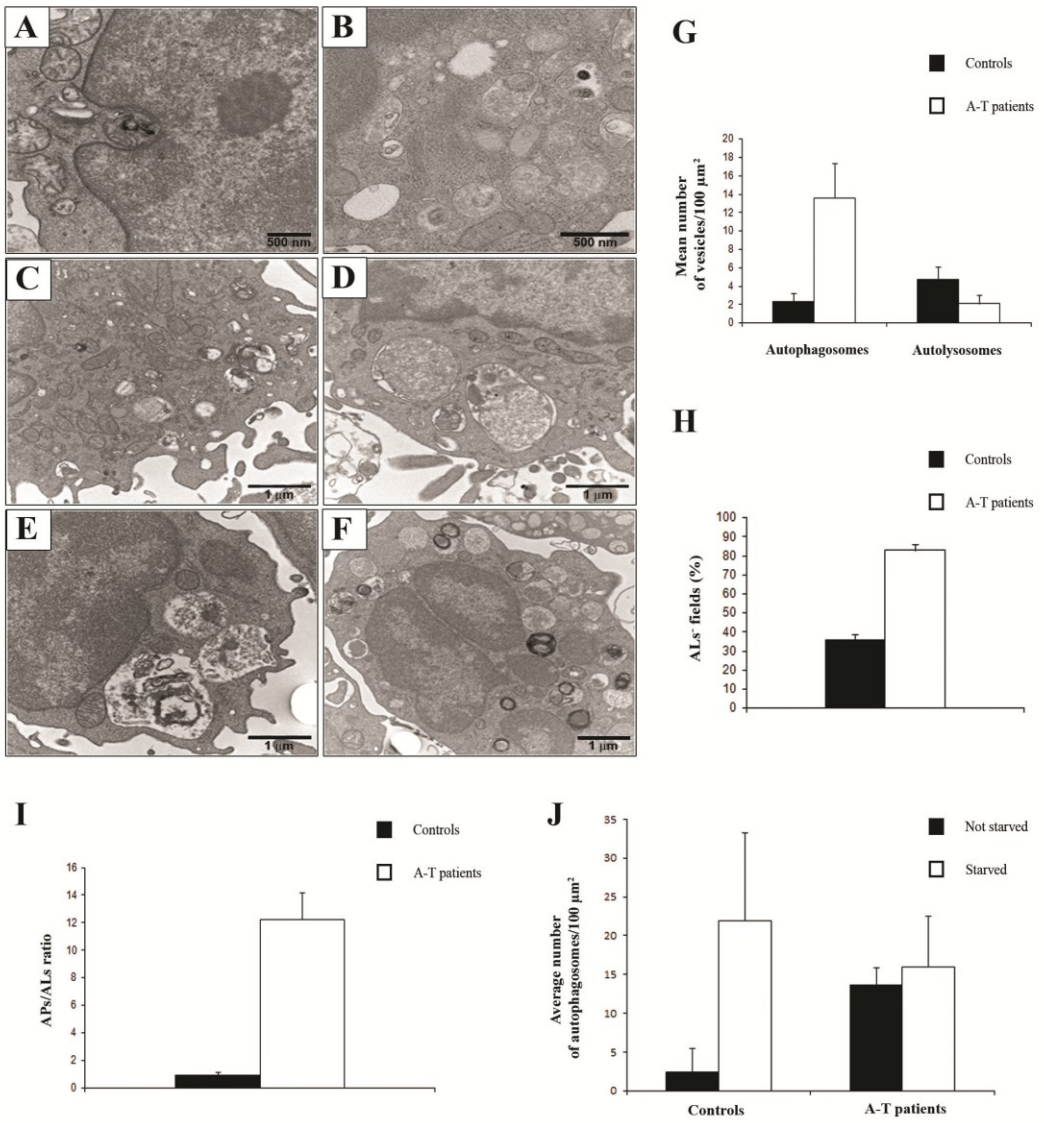

FIGURE 1. Lymphocytes from patients affected with A-T show accumulation of autophagosomes. **A-B**, Electron micrographs of freshly isolated lymphocytes from healthy controls in unstarved culture conditions or after serum starvation for 2 h showing the accumulation of autophagic vesicles (AVs). Scale bar: 500 nm. **C-F**, Electron micrographs of lymphocytes isolated from A-T patients at basal conditions showing a general engulfment of AVs. **D,** Autophagosomal vesicles (black arrows) with a double-membrane profile in the process of engulfment of targeted organelles. E, Autolysosomes (ALs) (white arrows) containing degraded material. **F,** Vacuolar structures in the late stage of the autophagolysosomal pathway. Scale bar: 1 µm. **G,** Number of autophagic vesicles/100 μm^2 in freshly isolated lymphocytes from healthy controls and A-T patients. $P = 0.03$. **H,** Percentage of ALs^- fields in fresh isolated lymphocytes from healthy controls and A-T patients. **I,** APs/ALs ratio for freshly isolated lymphocytesfrom healthy controls and A-T patients. $P< 0.05$. **J,** Number of autophagosomes/100 μm^2 in freshly isolated lymphocytes from healthy controls and A-T patients at basal conditions and after serum starvation for 2h. All data are expressed as mean ± S.D.

To further confirm that in A-T patients the autophagy was present at basal conditions, we tested the presence of LC3 puncta in lymphocytes from both A-T

patients and controls. Immunofluorescence analysis documented the presence of LC3 puncta in A-T patients but not in the controls, who showed a diffuse staining pattern of LC3 (Figure 2A). Moreover, as shown in Figure 2A, in few lymphocytes from A-T patients the LC3 marker co-localized with lysosome-associated membrane protein 2 (LAMP2), in contrast to the controls, where no merge signal between LC3 and LAMP2 was documented, thus indicating that in the patients, even at basal conditions, the autophagy was detectable. A quantitative analysis of LC3 positive cells, revealed that in A-T patients the percentage of cells with a puncta pattern was much higher than in the controls (82 *vs*12.25 %) (Figure 2B) and that in A-T patients about 4/5 of the total amount of LC3 molecule contained in the cells showed the puncta staining pattern (82% puncta *vs* 18% non-puncta; $p = 0.002$), thus resulting in a LC3 puncta cells/LC3 non-puncta cells ratio much higher in the patients than in the controls (6.56 *vs* 0.37) (Figure 2B).

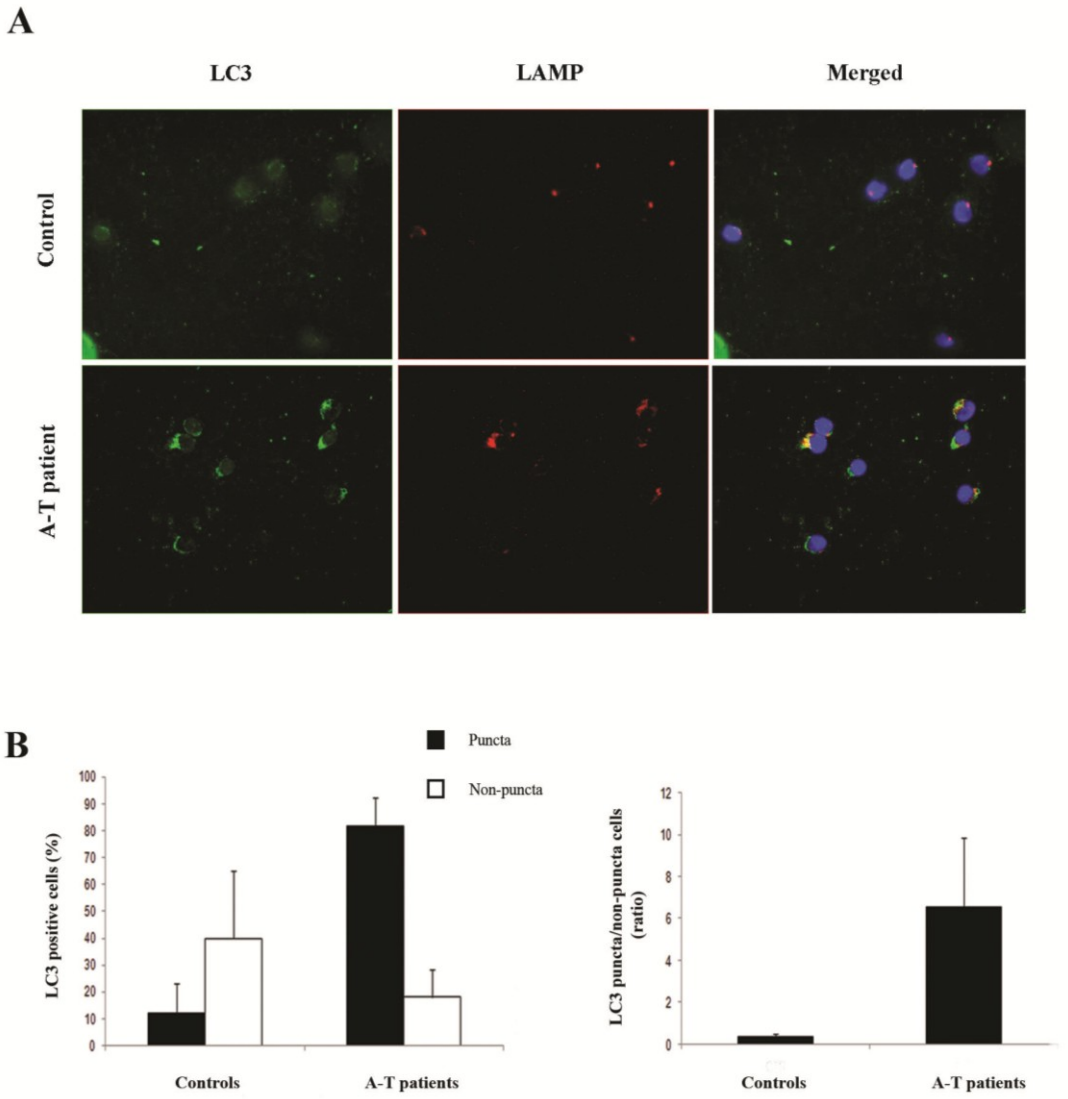

FIGURE 2. Autophagy is active in lymphocytes from A-T patients at basal conditions. **A,** Representative images of freshly isolated lymphocytes from healthy controls and A-T patients showing autophagosomes expressing LC3 marker (green punctate dots), lysosomes expressing LAMP2 marker (red dots) and autolysosomes (yellow dots). Cells were imaged by fluorescence microscopy. **B,** Percentage of autophagic vesicles positive for LC3 marker and LC3 puncta cells/LC3 non-puncta cells ratio in freshly isolated lymphocytes from healthy controls and A-T patients. Data expressed as mean ± S.E.

Since several genes are activated during autophagy, playing different roles in the different steps, we analyzed through quantitative real-time PCR the relative expression levels of an array of genes differentially involved in the process.

In A-T patients at basal conditions, mRNA expression level of *UVRAG*, involved in the biogenesis of APs, was higher than controls (Figure 3A), in keeping with the data obtained by TEM. The evaluation of the mRNA expression level of

genes involved in the lysosomes biogenesis and function (*CSTF*, *CSTB*, *CSTD*, *GBA*, *GUS*, *CD63*, *CSTA*, *CSTE*) revealed that, in A-T patients, at basal conditions, the expression of 6 out of the 8 genes analyzed was much higher than in the controls (Figure 3A), while under starvation conditions the expression was comparable, thus suggesting that in A-T, at basal conditions, the activation of the process was constitutively high. Taken together the results indicate that in A-T patients, the generation of both the APs and the lysosomes is very active. Moreover, the increased expression of 4 out of 5 cathepsins (*CSTB*, *CSTD, CSTA*, *CSTE*) and of the *GBA* hydrolase mRNAs in A-T at basal conditions indicates that lysosomal functionality is activated, as well (Figure 3B).

Since we observed an inappropriate APs/ALs ratio at the morphological studies in A-T, we also evaluated the expression level of *Vps11* and *Vps18* mRNAs, whose products are involved in vesicular trafficking and fusion. As shown in Figure 3C, at basal conditions, both *Vps11* and *Vps18* are less expressed in the patients than in the controls. The expression level of *Vps18* did not increase even after serum starvation, as compared to control expression.

To allow the encounter between APs and lysosomes, the vesicles have to be in the perinuclear zone. Since in the presence of nutrients, the lysosomes are linked to the plasmatic membrane through kinesins, we also evaluated the expression level of *KIF1Bβ*, *KIF2A*, *ARL8A*, *ARL8B* and *KIF3C*. As shown in the Figure 3D, at basal conditions, all the kinesins, and in particular the *KIF3C* molecule, were more expressed in A-T patients than in the controls, thus suggesting that the lysosomes are located in the peripheral zone of the cell, not favoring the fusion process between the vesicles.

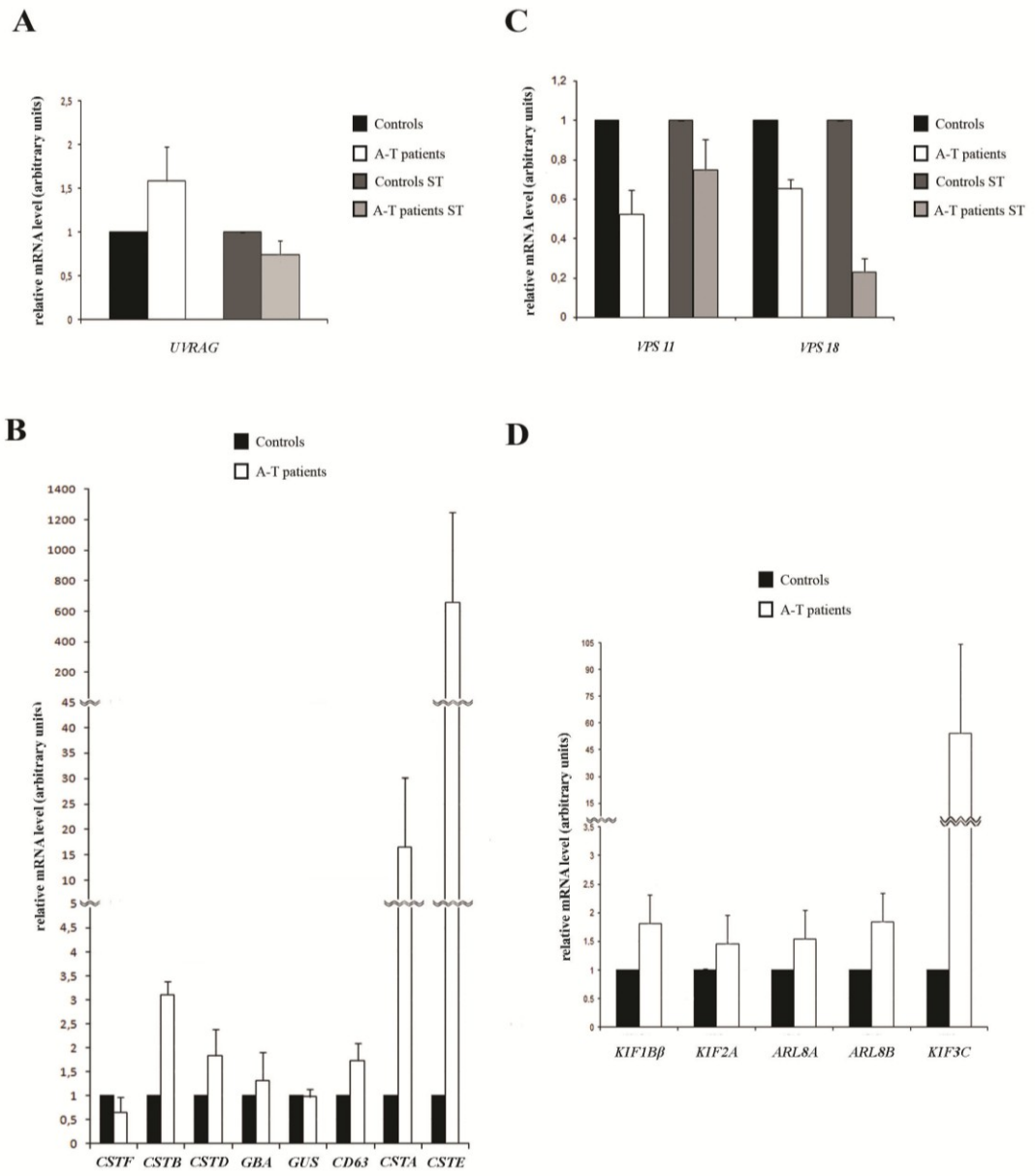

FIGURE 3. The accumulation of autophagosomes in lymphocytes from A-T patients is associated to a reduced expression level of Vps18 mRNA. **A-B,** Relative mRNA expression level of genes involved in the biogenesis of APs (*UVRAG*) and in the lysosomes biogenesis and function (*CSTF*, *CSTB*, *CSTD*, *GBA*, *GUS*, *CD63*, *CSTA*, *CSTE*), infreshly isolated lymphocytes from healthy controls and A-T patients, at basal conditions and after serum starvation for 2h. **C-D,** Relative mRNA expression level of genes involved in vesicular trafficking and fusion (*Vps11* and *Vps18*), at basal conditions and after serum starvation for 2h, and in lysosomal positioning in the cell (*KIF1Bβ*, *KIF2A*, *ARL8A*, *ARL8B* and *KIF3C*) in freshly isolated lymphocytesfrom healthy controls and A-T patients, at basal conditions. Data are expressed as mean ± S.E.

mTORC1 is an important sensor in regulating cell proliferation and inhibiting autophagy (35). Several pathways regulate its activation, which eventually results in the phosphorylation of downstream targets S6K (ribosomal protein 6 kinase), S6

(ribosomal protein 6) and 4EBP1 (eukaryotic initiation factor 4E-binding protein), involved in protein synthesis and survival. By contrast, ATM inhibits mTORC1. We investigated the phosphorylation status of mTOR (Figure 4A) and its downstream targets at basal conditions and after treatment with H_2O_2 at different time points (Figure 4B). We found that in A-T lymphoblastoid cell lines, the phosphorylation status of mTOR, S6K, S6and 4EBP1 proteins, was increased, in particular after 30' and 60' of treatment with H_2O_2, indicating that in A-T patients mTORC1 is active, and that autophagy mechanism is inhibited. Collectively, these data suggest that in A-T patients there is a lower ATM-mediated inhibition on mTORC1and of its downstream molecules, as compared to the control.

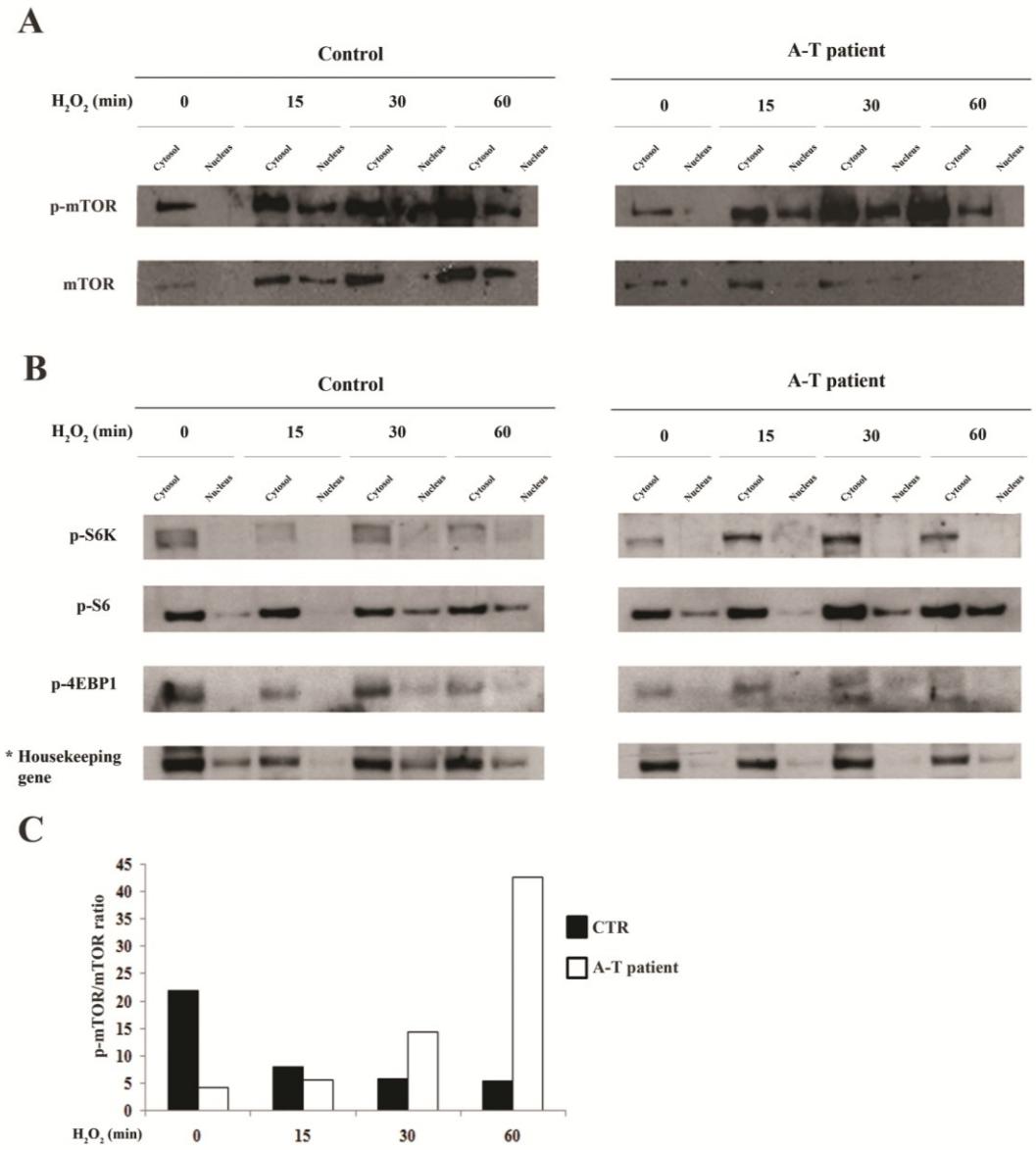

FIGURE 4. The activation of autophagy in A-T patients is not dependent on mTORC1 activity. Lymphoblastoid cells from A-T patients and controls were treated with 0.2 mM of H_2O_2 for different time points. **A,** Fractioned lysates were loaded for detection of mTOR and p-mTOR. **B,** Fractioned lysates were also loaded for detection of p-S6K, p-S6, p-4EBPI and housekeeping protein. **C,** p-mTOR/mTOR ratio in lymphoblastoid cells from A-T patients and controls.

SQSTM1/p62and LC3are the canonical markers to evaluate the autophagic process. In particular, SQSTM1/p62 translocates to the autophagosome formation site, where it is incorporated and then degraded (36). Its accumulation reveals an impaired autophagic flux (28). LC3-IIamount correlates with the number of autophagosomes, and is used as indicator of the autophagosome formation (37). Bafilomycin A1 (BAFA1), a specific inhibitor of vacuolar-type H^+ ATPase of the

lysosome, increases LC3-II levels, mainly during H_2O_2 induced stress, indicating that a normal flux occurs. We showed that in A-T patients lyphoblastoid cells, at basal conditions, SQSTM1 levels are accumulated as compared to controls (Figure 5A). Such increase was more evident in the presence of stress induced for 30' by H_2O_2 (Figure 5A). After treating for 1h with bafilomycin, we found that degradation of SQSTM1 was inhibited (Figure 5A), as expected. Furthermore, a synergic effect of H_2O_2 and BAFA1 treatment resulted in a remarkable increase of SQSTM1 level (Figure 5A). We also provided evidence that, in A-T lymphoblasts at basal conditions, Betamethasone pre-treatment is able to promote the degradation of SQSTM1, as shown in the Figure 5A. However, BMZ is ineffective in counteracting the effect on the molecule of the H_2O_2 induced stress (Figure 5A). By contrast, BMZ is able to counteract the inhibitory effect of the BAFA1 on SQSTM1 level (Fig 5A). In the control, BMZ *per se* promotes the degradation of SQSTM1. As expected, bafilomycin increased LC3-IIexpression under the condition of H_2O_2, induced stress. This increase was even more evident after BMZ pre-treatment. In particular, the overexpression of LC3-II after BAFA1 treatment, suggests an enhancement of the autophagic flux and a Betamethasone positive effect on the modulation of the autophagic process.

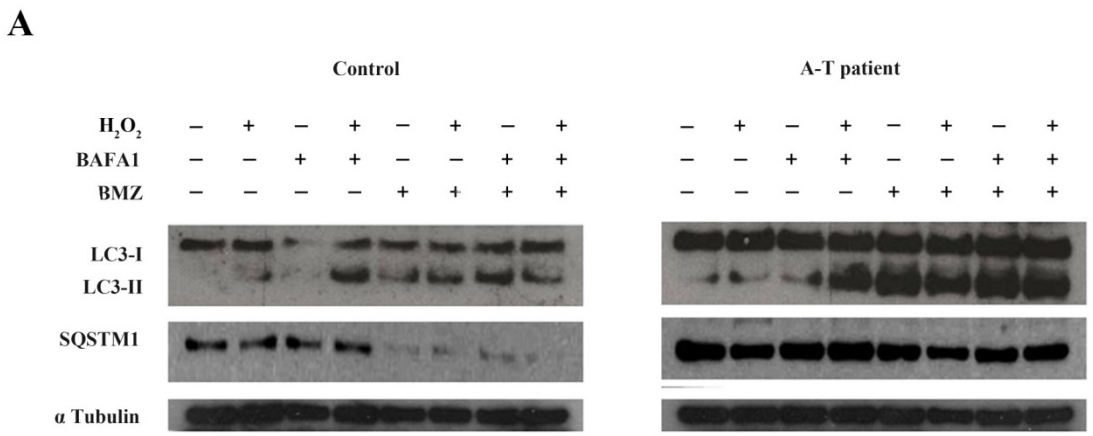

FIGURE 5. Betamethasone pre-treatment promotes autophagy inducing autophagosome formation and degradation. **A,** Lymphoblastoid cells from A-T patients and healthy controls were pre-treated or not with Betamethasone (BMZ) (80 nM) for 2h, and H_2O_2 (0.2 mM) for 0.5h in the presence or absence of 100 nM BAFA1 as indicated. Western blotting was performed to analyze the status of LC3, SQSTM1 and Tubulin.

In the present study we documented that lymphocytes isolated from A-T patients are characterized by an accumulation of APs, associated with a high expression of genes involved in the process of biogenesis and function of APs. This accumulation is not coupled to a parallel formation of autolysosomes, indicating the inappropriateness of the cell clearance apparatus, which ultimately results in the morphological appearance at the TEM of cytoplasmic engulfment of waste material.

Autophagy is a constitutive lysosomal catabolic process during which, cytoplasmic components, damaged proteins and entire organelles are degraded and recycled to generate building blocks for anabolic processes. The sequestration of cytosolic components within the APs is digested within the ALs after the fusion with the lysosomes. Autophagy is markedly induced by stress conditions, such as starvation, and has two major purposes: to recycle essential macromolecules and energy to be reused in conditions of nutritional scarcity or to clear cell from altered intracellular components (38). Neurons are post-mitotic cells, which require effective protein degradation to prevent accumulation of toxic aggregates. A growing body of

evidence now suggests that dysfunction of autophagy causes accumulation of abnormal proteins and/or damaged organelles. Such accumulation has been linked to synaptic dysfunction, cellular stress and neuronal death. Abnormal autophagy is involved in the pathogenesis of both chronic nervous system disorders, such as proteinopathies (Alzheimer's, Parkinson's, Huntington's diseases) and acute brain injuries (23). Known originally as an adaptive response to nutrient deprivation in mitotic cells, autophagy is now recognized as an arbiter of neuronal survival and death decision in neurodegenerative diseases (39).

Autophagy is a dynamic flux including vesicles biogenesis, fusion between APs and lysosomes and, eventually, contents degradation, and thus the increased number of APs herein observed may be due to an increased biogenesis or to a reduced degradation of the vesicles after the fusion with the lysosomes.

Moreover, it has recently been reported by Park et al. that autophagy is upregulated in response to DNA damage and that impaired cell cycle progression mediated through the checkpoint kinase 1 (Chk1), that allows DNA repair, affects the functionality of autophagy (40).

In keeping with the data obtained by TEM, documenting an accumulation of APs, we found in A-T lymphocytes, an increased expression of the *UVRAG* mRNA, which is specifically involved in the initial step of formation of APs, thus confirming, also at molecular level, that the stage of biogenesis of APs properly occurs in A-T patients. Moreover, the evaluation of CD63, which is expressed on lysosomal membrane, revealed an increased mRNA expression in A-T lymphocytes, indicating a proper lysosomal biogenesis.

An accumulation of APs may be due to abnormalities of regulatory molecules (41). Among the genes involved in lysosome functionality, cathepsins (CST),

lysosomal hydrolases belonging to the cysteine protease family A, B, D and F, and β-glucosidase and β-glucuronidase, help degrade unwanted intracellular or endocytosed proteins (30). In brain neurons of CST $D^{-/-}$ or CST $B^{-/-}$/CST $L^{-/-}$ mice, the lack of CST is responsible for the absence of protein degradation, which results in the accumulation of vacuolar structures, a feature similar to what observed in A-T lymphocytes. Similarly, in macrophages, the absence of CST S promotes an accumulation of APs (42), thus indicating a regulatory role for cathepsin family molecules. However, in A-T patients, even in unstimulated conditions, the mRNA expression levels of these proteases, *CSTB*, *CSTD*, *GBA*, *CSTA* and *CSTE*, are increased, thus indicating a constitutive hyperactivation of the lysosomal functionality. These data argue against the possibility that the accumulation of APs in A-T could be related to a reduced hydrolases activity.

The positioning of lysosomes plays an important role in that, only the lysosomes localized in the perinuclear area of the cell are able to fuse with APs and promote the cell clearance (33). The subcellular re-distribution of lysosomes mostly relies on their transport along microtubules mediated by the kinesins. The process ultimately leads to the formation of ALs where the degradation occurs. In this study, we found an overexpression in A-T cells of the kinesins KIF2A and KIF1B-β and the monomeric GTPase ARL8B and its close homologue ARL8A. A very impressive increase in KIF3C expression was also found. These results further support the inappropriateness in the process of lysosomal trafficking, which does not parallel APs formation, thus impairing the autophagic flux. By contrast, the A-T lymphocytes show a reduced expression of *Vps18* and *Vps11* mRNAs, whose protein products are involved in the vesicular trafficking and fusion between APs and lysosomes (34, 43). Both Vps11 and Vps18 are subunits of the Vps-C core complex also composed of

Vps16 and Vps33 (44). In eukaryotic organisms, the Vps-C complex acts as tethering factor in endosome- and lysosome-related vesicle fusion process. In particular, the down-regulation of *Vps18*, by knocking-down or by using anti-Vps18 antibody, blocks AP-lysosome and early endosome fusion, leading to an accumulation of APs and late endosomes in yeast (44). Additional data demonstrate that Vps18 plays a prominent role in the process (45). However, the immunofluorescence staining experiments revealed that, at a certain extent, the fusion process between APs and lysosomes in A-T cells did take place. Thus, we would argue that the reduced expression of *Vps11* and *Vps18* represents the limiting factor of the process, that does not allow an autophagic flux at a proper extent. Overall, the data herein reported indicate that the accumulation of APs may be due to the inefficacy of their degradation as a consequence of the reduced fusion process among APs and lysosomes in the perinuclear zone, determined byan altered regulatory control of the process. The increase in kinesins expression may be interpreted as an attempt to overcome the inappropriate APs-lysosome fusion process.

Taken together our data indicate that, in A-T lymphocytes, the fusion step between APs and lysosomes represents the limiting factor in the autophagy process. We identified the Vps18 protein as a potential checkpoint of the process.

Besides the nuclear activity of ATM, there is evidence that the molecule is also localized in the cytosol, within synaptosomal fractions (46), and, in particular, on the peroxisomes (47), where it induces pexophagy in response to ROS (48). It is also well documented that ATM, in the cytosol, has a role in the autophagy pathway (22). In fact, in the presence of elevated ROS levels, ATM activates the tumor suppressor TSC2, through LKB1/AMPK pathway, to inhibit mTORC1 and, in turn, induces autophagy (25). In this study, we documented in A-T lymphoblasts, an

increase of the phosphorylated S6K, S6and 4EBP1 proteins, which are mTORC1 substrates, and, in particular, under stress conditions. This is in keeping with the inhibitory effect of ATM on mTORC1 signaling**.** In addition, the increased p-mTOR/mTOR ratio in A-T patients, under stress conditions, further supports the ATM-mediated inhibition of mTORC1. Since in this study we found an accumulation of APs, this would imply that autophagy is a complex process and that the phase of APs formation is dependent on different regulatory pathways, which are not dependent on the cytosolic form of ATM. Whether the dysregulation of the process, herein described, directly depends on the absence of ATM mediated control of the pathway upstream to mTORC1 or rather to the defect in DNA repair remains to be further clarified.

In our recent studies, we documented transient improvement in A-T patients' neurological performance (5) and a paradoxical increase of proliferative response to common mitogens (6), after very low dosage of Betamethasone. Improvement of cerebellar symptoms during the steroid treatment was inversely correlated with the severity of cerebellar atrophy (7). Several studies have focused on the relationship between glucocorticoids and autophagy. It is known that GCs are able to induce autophagy in different cell types (49, 50), even though the explanation of the intimate molecular mechanism by which these drugs lead to this effect is not fully clear.

In the attempt to explain the beneficial effect of the steroids in A-T, we evaluated the effect of Betamethasone on molecular targets implicated in autophagy. We found that Betamethasone pre-treatment promoted the degradation of SQSTM1 protein, which was accumulated in A-T lymphoblastoid cells. The degradation of this protein is generally considered to parallel the progression of autophagy. We also found an accumulation of LC3-II protein after BMZ treatment, suggesting an

enhancement of the autophagic flux and a Betamethasone-mediated positive effect on the process.

In conclusion, we provide evidence of the inadequacy of the cell clearance apparatus in the cell from A-T patients, characterized by an accumulation of un-degraded APs. The molecular studies led us to identify in the fusion process between APs and lysosomes the limiting step,as checkpoint, in the overall process. The data herein reported have been submitted as *Brief Report* on *The Journal of Clinical Investigation.*

CHAPTER II

Severe Combined Immunodeficiencies

Severe combined immunodeficiency diseases (SCIDs) represent a heterogeneous group of rare genetic syndromes responsible for severe dysfunctions of the immune system, which share similar clinical manifestations. SCID is the most severe form of inherited primary immunodeficiency (PID) and its prevalence is approximately 1:100,000 live births, with a higher prevalence in males (1). SCIDs are difficult to recognize clinically because so many different infectious scenarios can occur. Without a functional cellular and humoral immune system SCID patients are susceptible to recurrent infections such as severe bacterial, viral, or fungal infections early in life and often present with interstitial lung disease, chronic diarrhea, and failure to thrive. In addition, some patients develop skin rashes, usually caused by maternal T cells transplacental engraftment during fetal life or by a wide autoreaction due to the activation of autologous T cells against skin components(2, 3).

Conventionally, SCIDs have been so far classified, on the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical immunological phenotypes related to a specific genetic defect. T cell–deficient but normal B cell (T−B+) SCID and bothT cell– and B cell–deficient (T−B−) SCID, in the presence or absence of NK cells (8) (Table 1).

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

Table 1. SCID classification

This classification helps in directing molecular studies toward a certain genetic alteration, since it is representative of the stage where the blockage occurs during the differentiation process (Figure 6).

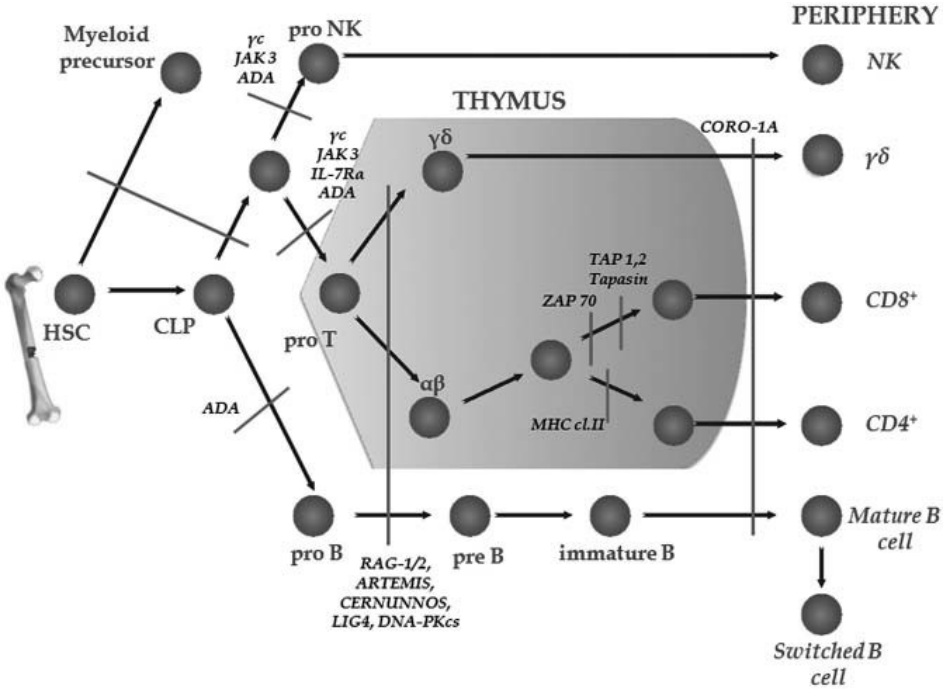

Figure 6. Genetic defects leading SCIDs (8).

Recently, due to the rapid growth of the knowledge in this field, many novel forms of SCIDs have been identified. These forms take into account several unusual and complex phenotypic presentations, due to non-hematopoietic alterations, leading to a pleiotropic phenotype charachterized by functional impairment of other organs different from immune system (8).

SCID patients often die during the first two years of life if appropriate treatments aimed at the reconstitution of the immune system, are not undertaken. Fortunately, the bone marrow transplantation (BMT) HLA-identical donor (RID) is the optimal treatment for patients affected with SCIDs (51). Moreover, the identification of the molecular causes of these forms of SCID has provided the basis for better prenatal and postnatal diagnosis, and opening the possibility to a potentially successful gene therapy.

2.1 Severe combined immunodeficiency-an update

Recently, Kwan *et al.*, on the basis of data obtained from 11 U.S. newborn screening programsin the general population, reported an incidence of SCID of 1 in 58,000 live-births, an incidence muchhigher than the previous estimate of one in 100,000 based on retrospective clinical diagnosis of SCID (52). The conventional classification for SCIDs, summarized in Table 1, traditionally considered as representative of the stage where the blockage occurs during the differentiation process, was, until a few years ago, very useful in directing molecular studies toward a certain genetic alteration. However, during the last years many new causative gene alterations have been identified with peculiar clinical and immunological phenotypes. In a few cases, the genetic alteration allows for a normal T cell differentiation program but compromises T cell functionality by affecting the initial or final phase of intracellular signaling. These functional T cell disorders are characterized by immune dysregulation and cancer predisposition, as well as infections. In addition, hypomorphic mutations in some SCIDs genes make possible the development of non functional oligoclonal T cells that are responsible for a complex of clinical conditions that may include hyperinflammation or autoimmunity. Many of the novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by functional impairment of organs different from primary lymphoid organs, which can make the diagnostic process very complex by standard methods. Taking this into account, the traditional international classification of SCIDs based on immunophenotype may no longer be optimal for clinical and research purposes (53), diagnostic criteria have to be continuously updated to take into account these unusual phenotypic presentations. In his work of 2014, Shearer emphasizes that currentlythere is no consensus among clinical

immunologists on how best to diagnose and treat these rare disorders. It is not surprising that an important clinical dilemma concerns the distinction of SCIDs from other diseases such as combined immunodeficiencies (CIDs). Recently, it was proposed that patients who exhibit an absence or a severe reduction of T cells (CD3+<300/μL), absence or severe reduction (<10% of the lower limit) of a proliferative response to phytohemagglutinin, or a maternal lymphocyte engraftment should be defined as having typical SCID (54). Moreover, the European Society for Immunodeficiency suggested as criteria for the diagnosis of CID the presence of one of the following parameters: one severe infection, an immunodysregulation disorder, cancer, familial CID associated with moderate age-related reduction of CD3+,CD4+, CD8+ T cells or of naive T cells. However,a cutoff to distinguish SCID from CID has not yet been well defined. Nowadays, hematopoietic stem cell transplantation (HSCT) still remains the only possible clinical approach for SCID patients, although, for two forms of SCIDs, and, probably in the future for other forms, gene therapy is considered for treatment.

The improvement in next generation sequencing technologies, have provided to the clinicians, a powerful tool to identify the genetic alterations responsible for PIDs of unknown genetic origin, even though in most cases the genetic cause still remains unknown.

A report on the biological and clinical features of SCID, paying attention to the most recently identified forms andto the unusual or extra-immunological clinical features, has been published as Review on *New York Academy of Science*, for the manuscript see below.

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Severe combined immunodeficiency—an update

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Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system. These diseases are life-threatening when the diagnosis is made too late; they are the most severe forms of primary immunodeficiency. SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken. Conventionally, SCIDs are classified according either to the main pathway affected by the molecular defect or on the basis of the specific immunologic phenotype that reflects the stage where the blockage occurs during the differentiation process. However, during the last few years many new causative gene alterations have been associated with unusual clinical and immunological phenotypes. Many of these novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by a functional impairment of several organs, which may lead to a considerable delay in the diagnosis. Here we review the biological and clinical features of SCIDs paying particular attention to the most recently identified forms and to their unusual or extra-immunological clinical features.

Keywords: severe combined immunodeficiency; SCID; primary immunodeficiency; nude/SCID; DiGeorge syndrome; cytokine; thymus

Introduction

Severe combined immunodeficiencies (SCIDs) are a group of inherited disorders responsible for severe dysfunctions of the immune system that lead to the absence or dysfunction of the T and B cells derived from the thymus gland and bone marrow, thus affecting both cellular and humoral adaptive immunity. Recently, Kwan *et al.*, on the basis of data obtained from 11 U.S. newborn screening programs in the general population, reported an incidence of SCID of 1 in 58,000 live-births, an incidence much higher than the previous estimate of one in 100,000 based on retrospective clinical diagnosis of SCID.[1] This group of diseases belongs to the most severe forms of primary immunodeficiency (PID), which are often fatal when the diagnosis is made too late.[2] Even though children with SCID appear healthy at birth, they are predisposed to severe bacterial, viral, and fungal infections as the maternal transferred antibodies decline. During the first year of life, failure to thrive, diarrhea, and oral candidiasis are common findings; *Pneumocystis jiroveci* may frequently cause a severe interstitial pneumopathy; and maternal engraftment of lymphocytes can cause graft-versus-host disease (GVHD).[3] SCID patients often die during the first two years of life if appropriate treatments to reconstitute their immune system are not undertaken.[4] For most patients, the only curative treatment is the allogeneic hematopoietic stem cell transplantation (HSCT).[5] Gene therapy offers a cure for two specific forms of SCID and, although other SCID forms may become amenable to this treatment in the future, it is likely that HSCT will continue to be used for the majority of SCID patients.[6]

Conventionally, SCIDs can be classified according either to the main pathways affected by the molecular defect or on the basis of the specific immunologic phenotype related to that genetic defect, as T cell–deficient but normal B cell (T^-B^+) SCID and both T cell– and B cell–deficient (T^-B^-) SCID, with a further subdivision depending on the presence or

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absence of NK cells (NK^+ or NK^-, respectively).[2] This classification, traditionally considered as representative of the stage where the blockage occurs during the differentiation process, was, until a few years ago, very useful in directing molecular studies toward a certain genetic alteration. However, during the last years many new causative gene alterations have been identified with peculiar clinical and immunological phenotypes. In a few cases, the genetic alteration allows for a normal T cell differentiation program but compromises T cell functionality by affecting the initial or final phase of intracellular signaling. These functional T cell disorders are characterized by immune dysregulation and cancer predisposition, as well as infections. In addition, hypomorphic mutations in some SCIDs genes make possible the development of nonfunctional oligoclonal T cells that are responsible for a complex of clinical conditions that may include hyperinflammation or autoimmunity. Many of the novel forms of SCID also show extra-hematopoietic alterations, leading to complex phenotypes characterized by functional impairment of organs different from primary lymphoid organs, which can make the diagnostic process very complex by standard methods. Taking this into account, the traditional international classification of SCIDs based on immunophenotype may no longer be optimal for clinical and research purposes[7,8]—diagnostic criteria have to be continuously updated to take into account these unusual phenotypic presentations. In his work of 2014, Shearer emphasizes that currently there is no consensus among clinical immunologists on how best to diagnose and treat these rare disorders. It is not surprising that an important clinical dilemma concerns the distinction of SCIDs from other diseases such as combined immunodeficiencies (CIDs). Recently, it was proposed that patients who exhibit an absence or a severe reduction of T cells ($CD3^+ < 300/\mu L$), absence or severe reduction (<10% of the lower limit) of a proliferative response to phytohemagglutinin, or a maternal lymphocyte engraftment should be defined as having typical SCID.[5] Moreover, the European Society for Immunodeficiency suggested as criteria for the diagnosis of CID the presence of one of the following parameters: one severe infection, an immunodysregulation disorder, cancer, familial CID associated with moderate age-related reduction of $CD3^+$, $CD4^+$, $CD8^+$ T cells or of naive T cells. However, a cutoff to distinguish SCID from CID has not yet been well defined.

A main aim of this review is to report on the biological and clinical features of SCID, paying attention to the most recently identified forms and to the unusual or extra-immunological clinical features (Table 2). An attempt to relate together pathogenetic mechanisms to specific clinical features is proposed (Table 1).

SCID due to defective survival of hematopoietic lineage precursors

Reticular dysgenesis (RD) is an autosomal recessive form of SCID characterized by both early myeloid lineage differentiation arrest and impaired lymphoid development.[9] It is considered the most severe form of SCID, accounting for less than 2%. A peculiarity of this disorder is the presence of sensorineural deafness. RD is caused by biallelic mutations in the adenylate kinase 2 gene (*AK2*), which cause the absence or the strong reduction of the expression of AK2 protein.[9,10] The syndrome is characterized by the absence of granulocytes and lymphocytes in peripheral blood. Compared to all the other forms of SCID, RD-associated neutropenia, which is unresponsive to granulocyte-colony stimulating factor (G-CSF), predisposes the patients to severe infections.[11] The only available treatment for RD is allogeneic HSCT, which indicates that the inherited defect is cellular and not linked to the micro-environment, as previously thought. Neutrophil differentiation abnormalities of RD patients are corrected by the restoration of AK2 expression in the bone marrow, thus confirming the specific role of AK2 in the development of the myeloid lineage.[12] Moreover, AK2 is specifically expressed in the stria vascularis region of the inner ear, which explains the sensorineural deafness observed in these individuals.[10] AK2 is localized in the mitochondrial intermembrane space where it regulates adenine nucleotide interconversion within the intermembrane space;[13] a very similar function is mediated by the cytoplasmatic enzyme AK1. The function of AK1/2 is classically described to be the maintenance of a constant concentration of adenine nucleotides and the monitoring of mitochondrial energy state through a fine mechanism of nucleotide sensing and signaling. The molecule also plays a central role in the control of apoptosis through the Fas-associated protein with death domain (FADD)

Table 1. New clinical phenotypes associated with old forms of nonsyndromic SCID/CID and new genetic defects

Gene defect	Old phenotype	New phenotype	Pathogenetic mechanism	Reference
AK2	Absence of granulocytes, severe lymphopenia sensorineural deafness	OS	Peripheral expansion of oligoclonal T lymphocytes	15
IL2RG (γc)	$T^-B^+NK^-$ SCID, leaky $T^+B^+NK^-$	Hodgkin like features, invagination and HLH	Not clear; maternal GVHD	55,56
JAK3	SCID, immune-dysregulation and autoimmunity	Selective $CD4^+$ T lymphopenia	Hypomorphic mutation associated with somatic chimerism	51
RAG	Severe hypogamma-globulinemia, marked reduction of T and B cells, OS, incomplete OS	Granulomatous lesions, EBV-related lymphoma, Idiopatic $CD4^+$ T lymphopenia with extensive chickenpox	Hypomorphic mutations	70
CORO1A	$T^-B^-NK^+$ SCID, severe postvaccination chickenpox, language delay, behavioral and cognitive impairment	EBV B cell lymphoproliferation	Not clear; null and hypomorphic mutations of *Coro1A* in mice are associated with defects in T cell survival and migration	79
FOXN1	Human nude/SCID	Eczematous rash, erythroderma, severe diarrhea and alopecia	Residual T cell development sustained by rudimentary thymus or extrathymic lymphoid sites	80
IL21R	NA	Cryptosporidiosis, chronic cholangitis and liver disease, abnormal IL-21 induced proliferation, defect of immunoglobulin class-switching, and NK cell cytotoxicity	Abrogation of IL-21 ligand binding, defective cytokine secretion	99
ZAP70	Selective $CD8^+$ lymphopenia and normal/elevated numbers of not functional $CD4^+$ T cells	Late onset disease, cutaneous, erythematous lesions, immune dysregulation erythroderma	Possible role of hypomorphic mutations on T lymphocytes effector and suppressive function	113
MALT1	NA	CID	Abnormal IL-12 production, failure of IκBα degradation	114
BCL10	NA	Profound T and B memory cell deficiency, severe hypogammaglobulinemia	Impairment of NF-κB pathways	115
CARD11	NA	CID	Abnormal IL-12 production, T_{reg} cells deficiency	101
TTC7A	NA	CID-MIA	Defective thymopoiesis	116
LCK, UNC119	NA	$CD4^+$ lymphopenia, restricted T cell repertoire, immune dysregulation	Impaired TCR signaling	122
IKBK2	NA	Mycobacterium avium and tuberculosis infections, neurological impairment, hypogammaglobulinemia, normal T cells count with absence of T_{reg} and γ/δ T cells	Impairment of IKK2–NF-κB signaling	124

NOTE: OS, Omenn syndrome; HLA, hemophagocytic lymphohistiocytosis, GVHD, graft versus host disease; MIA, multiple intestinal atresia; NA, not applicable.

Table 2. Pathogenetic mechanisms of SCID

Pathogenetic mechanism	Defect	Phenotype	Inheritance
Defective survival of haematopoietic precursors	AK2	$T^-B^-NK^-$	AR
Toxic metabolite accumulation	ADA	$T^-B^-NK^-$	AR
	PNP	$T^-B^+NK^-$	AR
Cytokine signaling anomalies	IL-2RG	$T^-B^+NK^-$	XL
	JAK3	$T^-B^+NK^-$	AR
	IL-7RA	$T^-B^+NK^+$	AR
V(D)J recombination and TCR abnormalities	RAG1/RAG2, Artemis, DNA-PKcs, Cernunnos, LIG4	$T^-B^-NK^+$	AR
TCR abnormalities	CD45	$T^-B^+NK^+$	AR
	CD3ε, δ, ζ	$T^-B^+NK^+$	AR
	CORO1A	$T^-B^-NK^+$	AR
Thymic abnormalities	FOXN1	$T^{-/low}B^+NK^+$	AR
	DiGeorge sindrome	$T^-B^+NK^+$	De novo or AD

and caspase 10 pathways.[14] Omenn syndrome (OS), resulting from residual development and peripheral expansion of oligoclonal T lymphocytes, has recently been described in a patient with RD due to missense mutation in *AK2*.[15] OS is a clinical condition characterized by generalized skin rash, hepatomegaly, splenomegaly, lymphadenopathy (similar to that which occurs in SCID patients with detectable $CD3^+$ T cells), absent or low T cell proliferation to common antigens, and no maternal engraftment. Increased IgE serum levels and eosinophil count are also common features. In rare patients with RD, no mutations in *AK2* have been found, suggesting a potential role for other molecules involved in this pathway. For instance, a similar phenotype has been described in a murine models either deficient for growth factor independence-1 (Gfi-1) or transgenic for expression of Gfi-1b nucleoproteins, suggesting a role for these two factors in the pathogenesis of RD.[16]

SCID due to accumulation of toxic metabolites

Adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase (PNP) deficiency are inherited disorders of the purine metabolism characterized by abnormal accumulation of toxic nucleoside products.[17] ADA deficiency is responsible for a T cell–, B cell–, and NK cell–deficient ($T^-B^-NK^-$) form of SCID associated with thymic hypoplasia and absence of lymphocyte proliferative response. Before the introduction of newborn screening, the incidence of this autosomal recessive disorder was estimated to be between 1:375,000 and 1:660,000 live births.[18] However, a recent trial on a population-based neonatal screening revealed that the incidence of ADA-SCID is much higher, and closer to 1:50,000.[19] The ADA gene of 12 exons is located in a 32 kb region on chromosome 20q13.11. Several genetic alterations, with more than seventy mutations, have been identified in ADA-SCID patients.[20] The product of *ADA* is an ubiquitous enzyme that catalyzes the irreversible deamination of adenosine (Ado) and deoxyadenosine (dAdo) to inosine and deoxyinosine, respectively. Despite ADA protein being present in virtually every cell of the human body, it is particularly expressed in the lymphoid system, especially in the thymus, where it plays a key role in its differentiation and maturation. The absence of ADA activity is responsible for a massive accumulation of Ado and dAdo, in particular in thymocytes, lymphocytes, and erythrocytes.[17,21] dAdo phosphorylation by nucleoside kinases leads to the production of deoxynucleotide triphosphates (dATP) whose accumulation, altering lymphocyte signaling pathways and serving as a danger signal, may cause the severe lymphopenia observed in ADA deficiency. Another alternative pathogenic mechanism proposed is the inhibition of *S*-adenosylmethionine–mediated transmethylation reactions required for

cell viability and normal differentiation.[22] By the first 6 months of age up to 80% of patients show multiple recurrent opportunistic infections that rapidly may become fatal and hypoplasia or apparent absence of lymphoid tissue. However, in the remaining patients, a late-onset phenotype, presenting at two or three years of life, or even later,[23] has been reported. These patients may also present with autoimmune diseases and usually exhibit a milder T cell immunodeficiency, which gradually progresses. Owing to its ubiquitous expression normally, ADA deficiency can affect several organs, leading to the development of skeletal alterations, such as anterior rib cupping, scapular spurring, and pelvic dysplasia, which can be reversible with appropriate therapy. In addition, pulmonary alveolar proteinosis, probably caused by a surfactant metabolism defect, and hepatic, gastrointestinal, and neurological disorders, mainly due to Purkinje cell damage, may be found. Bone marrow hypocellularity and myeloid dysplasia also have been observed in some ADA-deficient patients; in others, renal impairment.[24,25] A genotype–phenotype correlation has been documented and, in particular, severity of disease seems to correlate with residual ADA activity and the types of substrates that accumulate.[26] The therapeutic approach currently available for this particular form of SCID includes three options: enzyme replacement therapy with polyethylene glycol-modified bovine adenosine deaminase, HSCT, or gene therapy.[27–29] The use of dried blood spot samples tested by tandem mass spectrometry has been recently proposed as part of a neonatal program of screening in several countries.

Purine nucleoside phosphorylase gene (*PNP*) mutations result in an extremely rare autosomal recessive disorder accounting for 4% of all form of SCIDs.[30] Autoimmunity, recurrent infections, failure to thrive, and neurologic dysfunction are some of the main features of PNP deficiency. *PNP* maps to chromosome 14q13 and encodes a protein that catalyzes the phosphorolysis of guanosine, deoxyguanosine, inosine, and deoxyinosine, to their respective purine bases.[17,31,32] Mutations in the PNP pathways result in elevated deoxyguanosine triphosphate storage and in T cell toxicity due to the inhibition of the mechanisms of DNA synthesis and repair, resulting in an increased sensitivity to DNA damage and apoptosis, especially in T lymphocytes during selection within the thymus.[33] T cell defects typically become evident by the first year of life, with a milder phenotype than what is normally seen in ADA deficiency. PNP deficiency can be suspected when lymphopenia is associated with reduced PNP enzymatic activity in red blood cells in a patient with recurrent respiratory infections and other typical manifestations.[34] Low serum uric acid (hypouricemia) is usually found, although PNP deficiency should not be ruled out if patients do not exhibit it. The immunodeficiency in these patients is progressive, since the severe T cell deficiency usually appears after the second year of life and is characterized by a normal B cell compartment. Among the neurological disorders associated with PNP deficiency, ataxia, developmental delay, and spasticity have been described. Autoimmune diseases observed include hemolytic anemia and sclerosing cholangitis,[35] and in some patients megaloblastic or dysplastic bone marrow has been described.[33]

SCID due to cytokine signaling anomalies

Cytokines are soluble regulators of immune system homeostasis. Alterations of their signaling are implicated in the pathogenesis of the major SCIDs. In particular, SCIDs caused by defects of the common gamma chain (γc), Janus kinase 3 (JAK3), or the IL-7 receptor α chain (IL-7Rα) are prototypic cytokine-associated disorders, accounting for 67–74% of all cases of SCIDs.[36,37]

Mutations of γc gene cause X-linked SCID (X-SCID), one of the most common forms of SCID, accounting for 50% of all cases. The γc gene (*IL2RG*) maps to chromosome Xq13.1 and encodes a transmembrane protein that is a component of several cytokine receptors, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all critical for lymphocyte development and function.[38] The γc interacts with the intracellular tyrosine kinase JAK3, which acts as a transducing element[39] indispensable for cell growth and control of hematopoietic cell development. Evidence indicates that γc is widely expressed in non-hematopoietic cells as well, even though its function in these cells has not yet been clearly elucidated. It has been reported that γc is implicated in the growth hormone receptor signaling, suggesting the existence of a subtle interaction between endocrine and immune systems.[40–44]

JAK3, mainly expressed in lymphoid and myeloid cells, is essential for the differentiation of

hematopoietic precursors;[45–47] its deficiency is responsible for an autosomal recessive SCID. Molecular alteration of JAK3 may affect any of its functional domains and results in a $T^-B^+NK^-$ form of SCID, with a clinical phenotype similar to that observed in γc deficiency.[48] The immunological phenotype is due to the key role of γc/JAK3 signaling in both early T and NK cell, but not B cell, differentiation programs. However, B cell–intrinsic abnormalities, such as impaired class switch recombination and defective antibody production, have been documented. The identification of IL-7R–deficient SCID patients with a selective T cell defect[37] implies that the T cell defect observed in SCID due to mutations of γc/JAK3 results from defective IL-7 signaling. The ability of IL-15 to drive NK cell development[49] explains the lack of NK cells in γc/JAK3-deficient patients as a consequence of defective IL-15 signaling.[50] The molecular basis of the B cell functional abnormalities in patients with γc/JAK3 deficiency is probably linked to a defect in IL-21 secretion, a cytokine involved in proliferation, Ig isotype switching, plasma cell generation, and antibody secretion through activation of the JAK/STAT pathway.

Recently, hypomorphic mutations in JAK3 associated with somatic chimerism have been reported in a patient with predominant $CD4^+$ lymphopenia.[51] This observation suggests that hypomorphic mutations and/or somatic chimerism in other genes, which usually cause a SCID phenotype, eventually could be implicated in selective $CD4^+$ lymphopenia. Individuals with mutations that result in the production of a small amount of gene product or a protein with residual activity are less frequently seen. These individuals may have an atypical "leaky" disease characterized by $T^+B^+NK^-$ phenotype that is associated with immune dysregulation and autoimmunity, rashes, splenomegaly, gastrointestinal malabsorption, and/or short stature;[52,53] a few patients have presented with an OS phenotype,[54] which is characterized by elevated IgE, erythroderma, and an expansion of cells with a lymphocyte profile.

A peculiar extranodal lymphoproliferative disorder characterized by a polymorphous $CD20^+$ B lymphocyte infiltrate, resembling Hodgkin Reed-Sternberg cells, has also been observed in two patients affected with X-SCID.[55] Recently, a novel mutation in exon 5 of the γc gene has been reported that causes a classical severe immunological phenotype associated with invagination and hemophagocytic lymphohistiocytosis (HLH).[56] The HLH phenotype, previously described in two other cases with γc gene mutations,[57] is probably explained by maternal GVHD, and highlights the need for a fine-grained evaluation of the immunological phenotype and associated genotypes in patients with HLH.[58] As for the mechanism by which maternal engrafted T cells may be responsible for HLH in such cases, it is reasonable to hypothesize that unchecked T cell dysregulation of $CD8^+$ cells, activated by alloantigens, may result in cytokine hypersecretion and massive macrophage activation, eventually leading to hemophagocytosis.

The mutations of IL-7Rα gene (*IL7R*) cause a $T^-B^+NK^+$ SCID with an autosomal recessive transmission that is responsible for 10% of all SCIDs. The human *IL7R* maps to chromosome 5p13.2 and encodes for a protein[59] that is a component of two cytokine receptors, namely IL-7R and thymic stromal lymphopoietin receptor (TSLPR). Following the binding of IL-7 to IL-7R, JAK1 (coupled to IL-7Rα) and JAK3 are activated, which induces the phosphorylation of IL-7Rα, the recruitment of STAT5, and phosphatidylinositol 3-kinase (PI3K) at the receptor signaling apparatus. STAT5 molecules dimerize and translocate to the nucleus, leading to the transcription of IL-7–dependent genes. PI3K induces Akt activation, which prevents cell death through inhibition of Bad and regulates the kinase activity of Tor, eventually leading to the induction of several nuclear targets, including nuclear factor of activated T cells (NF-AT), NF-κB, and cyclin D1. Finally, activation of the Ras/MAPK/ERK pathway results in the induction of other nuclear targets, such as c-Myc, STAT1/3, and the Ets transcription factors. IL-7R is almost exclusively expressed by cells of the lymphoid lineage and is involved in thymocyte survival and maturation, particularly during $CD8^+$ positive selection.[60]

TSLPR, expressed mainly on monocytes, dendritic cells (DCs), and some types of T lymphocytes, is able to activate JAK2/STAT5 pathway, although this does not lead to cell proliferation. Human TSLP acts primarily on DCs, promoting DC-mediated expansion of $CD4^+$ T lymphocytes that acquire a memory T cell phenotype. The clinical phenotype of this form of SCID is quite heterogeneous and includes peculiar features such as OS,[61] cytopenia,[62]

severe and unresponsive cytomegalovirus (CMV) infection, or diarrhea of probable viral origin.[61]

SCID due to V(D)J recombination and TCR abnormalities

V(D)J recombination is a complex process that occurs in early B and T cell development. It is responsible of the introduction of site-specific DNA double strand breaks (DSBs) by the recombination activating genes (RAG) 1 and 2.[63,64] The cleavage of the hairpin and the joining of these segments requires the DNA nonhomologous end-joining (NHEJ) DNA repair factors, which generate the diversity through recombination of the V, D, and J segments and junction.

NHEJ also plays a role in preserving the genomic stability of cells exposed to X-ray DNA damage. Consistent with these functions, it is not surprising that mice lacking NHEJ components exhibit a SCID phenotype and radiosensitivity (RS), a phenotype referred to as RS-SCID. In humans, several mutations in NHEJ genes have been identified, including mutations in genes for DNA ligase IV (*LIG4*), XLF/Cernunnos (*NHEJ1*), DNA-PKcs (*PRKDC*), and Artemis (*DCLREIC*), that are associated with SCID.[64–66] Of note, the increased radiosensitivity peculiar to these forms of SCID can be used as a diagnostic tool.[67,68]

Owing to the essential role of RAG1/RAG2 genes in V(D)J recombination, mutations of *RAG1* and/or *RAG2*, associated with partial protein expression and limited production of T and B cells, have been associated with a $T^-B^-NK^+$ SCID, OS, and autoimmunity.[69] Hypomorphic RAG gene mutations have also been described in patients with granuloma formation[70] and EBV-related lymphoma.[71] Since different clinical phenotypes have been associated with similar RAG mutations resulting in the same biological effect, a complex pathogenetic mechanism, based not only on the residual recombinase activity but also on the type and the moment of antigenic pressure has been postulated.

Artemis deficiency causes T cell maturation and B cell differentiation arrest at the pre-B cell checkpoint, resulting in a $T^-B^-NK^+$ SCID.[68] DNA-PKcs is involved in Artemis regulation and activation by both phosphorylation and complex formation, thus regulating enzymatic activities critical for V(D)J recombination.[64,72] Deficiency of DNA-PKcs causes a phenotype similar to Artemis deficiency.

The deficiency of XLF/Cernunnos causes a $T^-B^-NK^+$ SCID phenotype associated with microcephaly.[73] In particular, the phenotype is characterized by a progressive decrease of B cells and the presence of only memory T cells. Crystallography studies showed that XLF/Cernunnos is a component of the LIG4/XRCC4 complex, which exerts a role in aligning the two DNA ends in the DNA repair complex machinery. Deficiency of LIG4 is responsible for facial dysmorphisms, microcephaly, and variable forms of PID, ranging from SCID/OS to hypogammaglobulinemia or moderate defects in T and B cell functions.[74]

Gene mutations that abrogate early TCR signaling are associated with profound abnormalities of T lymphocyte development and function. CD45 (leukocyte common antigen) is a transmembrane tyrosine phosphatase involved in both TCR signaling and T cell development within the thymus and B cell development and maturation. CD45 deficiency is responsible for a very rare form of $T^-B^+NK^+$ SCID in which lymph nodes lack germinal centers.[75] Despite a normal monocyte numbers, T lymphocyte numbers are considerably decreased, with normal expression of TCR γδ chains but a reduction of TCRαβ$^+$ cells. B cells, even though nonfunctional, are increased in number.

CD3 is a multimeric complex involved in TCR signaling and required for T cell differentiation. Defects of the complex can involve all the chains, resulting in a $T^-B^+NK^+$ phenotype. Alterations of the subunits epsilon (CD3ε), delta (CD3δ), and zeta (CD3ζ), have been reported in patients with severe forms of SCID, while alterations of the CD3γ have been associated with a more benign course. These disorders are rare and inherited as autosomal recessive SCIDs. Some mutations can allow residual T cell maturation, even though the cross-talk between thymocytes and thymic epithelial cells may be impaired, thus compromising central tolerance and regulatory T cell (T_{reg}) development. Autoimmune manifestations, including autoimmune hemolytic anemia, vitiligo, Hashimoto's thyroiditis, autoimmune enteropathy, Evans syndrome, autoimmune hepatitis, and nephrotic syndrome are frequently observed in such patients.[76]

Coronin-1A is important for regulation of actin polymerization of cytoskeleton and essential for T cell migration from the thymus to the secondary lymphoid organs.[77] The human coronin-1A gene

(*CORO1A*) maps to chromosome 16p11.2 and encodes a highly conserved 57-kDa actin-binding protein expressed in both hematopoietic and immune cells. Coronin 1A–deficient neutrophils of mice have a normal adherence, membrane dynamics, migration, phagocytosis, and oxidative burst; dendritic cells are similarly not impaired. However, coronin 1A–deficient mice exhibit T cell lymphocytopenia and a normal number of B and NK cells, thus confirming its prominent role in T cell homeostasis and TCR signaling. In humans, deficiency of coronin 1A is associated with the absence of peripheral T cells.[78] However, different from other SCIDs due to other genetic alterations, a normal size thymus has been observed in the context of coronin 1A deficiency.[79] Hypomorphic *CORO1A* mutations have been associated with aggressive Epstein Barr virus–associated B cell lymphoproliferation, occurring at an early age.[79]

SCID due to thymic abnormalities: from DiGeorge syndrome to nude/SCID

The prototype of athymic disorders caused by abnormalities of the stromal component of the thymus—the primary lymphoid organ for T cell differentiation—is the nude/SCID syndrome, described in humans in 1996.[80] This form of SCID is the only one not primarily related to an intrinsic abnormality of the hematopoietic cell, but rather to a defect in hematopoietic cell–supporting thymic epithelial cells.[81–83] This human SCID is the equivalent of the murine nude/SCID phenotype described in 1966, although in humans the phenotype is more severe. It is one of the rarest forms of SCID, and only three mutations have been associated thus far with nude/SCID.[84] The gene responsible for the disease in humans is *FOXN1*, located on chromosome 17,[85] which encodes a member of the forkhead/winged helix class proteins; this same gene is mutated in the same type of SCID in mice and rats. Forkhead/winged helix proteins is a large family of transcriptional factors implicated in several biological processes governing development, metabolism, cancer, and aging. FOXN1 is mainly expressed in the epithelial cells of the skin and thymus, where it plays a role in maintaining the balance between growth and differentiation. Thymic epithelial cell precursors require FOXN1 for full differentiation into cortical and medullary thymic epithelial cells capable of supporting T cell development. In epithelial cells, FOXN1 contributes to keratinocyte proliferation and differentiation in hair follicles, and to the development of the choroid plexus epithelium; this could explain the major features that characterize patients with nude/SCID, namely the absence of the thymus, with a severe T cell defect (though normal B and NK cells) and abnormal skin development, including congenital alopecia and nail dystrophy. The syndrome belongs to the $T^-B^+NK^+$ subgroup of SCIDs.[81] Usually, there is a significant reduction of $CD3^+CD4^+$ T helper lymphocytes, while the number of $CD3^+CD8^+$ T cells is less reduced. Functionally, there is a severe impairment of the proliferative response to mitogens, as found in the other forms of SCIDs.

The mutations described in nude/SCID cause a complete absence of functional FOXN1 protein. The first known mutation identified in humans, R255X, truncates the protein before the start of the forkhead domain, while a second mutation, R320W, leads to a substitution in the protein's DNA binding domain. A third mutation, c.562delA, results in a frameshift and premature truncation of the protein (p.S188fs) after the first 24 amino acids of the forkhead domain. The disease is inherited as an autosomal recessive trait. Heterozygous patients show minor ectodermal anomalies, such as nail dystrophy and, in particular, leukonychia or koilonychia (spoon nail).[86,87] Recent studies support a role for FOXN1 as cofactor in the development and differentiation of the central nervous system.[88]

Bone marrow transplantation (BMT) to treat this nude/SCID, despite the favorable clinical course, often results in a progressive decline of the $CD4^+$ T cell compartment[89] owing to the fact that a normal thymus is necessary for the generation of the $CD4^+$ naive subset. Conversely, the production of $CD8^+$ naive lymphocytes after BMT is less thymus dependent and even occurs in nude/SCID patients. In addition, a recent study showed the presence of T lymphocytes in a $FOXN1^{-/-}$ human fetus, suggesting partial T cell ontogeny in a thymus- and FOXN1-independent process.[90] Thymus transplantation has been shown to lead to immune reconstitution in two nude/SCID patients affected with disseminated Bacillus Calmette-Guérin infection and cytopenia.[91]

Before the identification of human nude/SCID, the DiGeorge syndrome (DGS) was long considered the model of a severe T cell differentiation defect. DGS is a complex disorder that typically

comprises T cell deficiency due to thymic hypo/aplasia, hypoparathyroidism, conotruncal cardiac defects, facial abnormalities, cognitive defects, speech delay, other birth defects, and gastrointestinal disorders.[92] Deletion of 22q11.2 is the most frequent chromosomal change associated with DGS,[93] with an incidence of one in 4000–5000 live births. The alteration is inherited in a familial autosomal dominant pattern in 8–28% of the cases.[94] Most patients have a deletion of 3 Mb that includes about 30 genes, while in 8% of the cases a smaller deletion of 1.5 Mb containing 24 genes is detected. No specific genotype–phenotype relationship has been documented. Both deletions include the gene T-box transcription factor 1 (*TBX1*), which seems to be necessary for normal development of the thymus and parathyroid, the large arteries of heart, and the muscles and bones of face and neck. Thymic hypoplasia, responsible for the thymic dysfunction, is observed in more than 80% of patients. The syndrome may be associated with variable T cell deficiencies, ranging from close to normal T cell numbers and functions, to complete DGS with a $T^-B^+NK^+$ SCID-like phenotype accounting for less than 1% of DGS.[95] Recently, a phenotype characterized by a $T^-B^-NK^+$ SCID has been described in two DGS patients with a concomitant Artemis deficiency.[96] Patients with complete DGS, like other infants with SCID, suffer from severe opportunistic infections and exhibit a high risk of acquired GVHD if transfused. Furthermore, a few patients affected with an atypical complete DGS have mature T cells derived from maternal engraftment or oligoclonal expansion of memory T cells responsible for a severe inflammation. These patients may develop an OS, characterized by erythrodermia, enteropathy, and lymphadenopathy. On the other hand, there are also subjects carrying the deletion who only have a mild phenotype. Some patients diagnosed as 22q11.2DS in early childhood remain clinically asymptomatic and exhibit only minimal immune alterations. Increased prevalence of atopic and autoimmune diseases has been reported in patients with partial deletion syndrome.[97] While normal B, NK, and T cell numbers are frequently observed in 22q11.2DS individuals, sometimes, a decrease of $CD4^+$ and $CD8^+$ T lymphocytes may be found[97] due to lower thymic output of the naive T cell subset, oligoclonal T lymphocyte expansion,[98] or altered T cell differentiation. These observations can be explained by the dysregulation of peripheral T cell homeostasis due to a defect in IL-7 signaling, crucial for T lymphocyte survival and expansion and for homeostasis of the naive $CD4^+$ T cell pool. Indeed, subjects with 22q11.2DS show a significant decrease of $CD3^+$ T lymphocytes expressing IL-7Ra; adults have accelerated conversion of naive to memory cells, shorter telomeres, and a defect in the variability of the TCR repertoire.[98]

A DGS phenotype has been described in patients carrying a 10p deletion, the clinical features being almost undistinguishable from 22q11.2DS. Even though low numbers of T cells, reduced immunoglobulin,[80] and thymus hypoplasia have been observed in 28% of such patients, none have been affected with a severe SCID-like phenotype.[95]

SCID/CIDs associated with syndromic features

According to the International Union of Immunological Societies (IUIS), there are forms of PID associated with highly pleomorphic extra-immunological features responsible for complex syndromes with a genetic basis. Typical features of these syndromes comprise peculiar facial dysmorphism, growth delay, microcephaly, and ectodermal abnormalities. While an increased susceptibility to autoimmunity and (occasionally) cancer associated with the depletion of other blood cell lines is frequently reported, an increased susceptibility to infections is usually less frequent, and its clinical relevance is lower than in other PIDs. The pathogenetic mechanism resides in the involvement of several genes expressed in multiple cell lines, genes responsible for both ontogenesis and maturation of the immune system, as well as morphogenesis and organogenesis of other organs. Some of these conditions may be associated with a SCID/CID phenotype. Several syndromes are included in this group (Table 3), such DGS and CHARGE syndrome. Patients with CHARGE syndrome exhibit variable grades of immune defects, ranging from severe to mild T cell lymphopenia and abnormal T cell functionality, sometimes associated with hypogammaglobulinemia.[102] The incidence of SCID in patients with CHARGE is unknown, even though it may be, as in DGS, rare.[103] These patients, whose clinical phenotype is characterized by coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, and

Table 3. Peculiar clinical and laboratory findings in the main genetic syndromes which in a few cases may be associated with a SCID/CID phenotype

Disorder	Genetic defect	Clinical phenotype	Immunological features
CHARGE syndrome	*CHD7*	Coloboma, hearth defect, atresia choanae, retarded growth and development	$T^-B^+NK^+$ SCID, OS, T cell lymphopenia, hypogamma-globulinemia
Cartilage–hair hypoplasia (CHH)	*RMRP*	Short limb with metaphyseal dysostosis, sparse hair, neural dysplasia of intestine	T cell lymphopenia, hypogammaglobuline-mia, antibody deficiency
Schimke immuno-osseous dysplasia	*SMARCAL1*	Short stature, IUGR, spondiloepiphyseal dysplasia	T cell lymphopenia, bone marrow failure
Hyper IgE syndrome	*PGM3*	Short stature, brachydactyly, facial dysmorphism, intellectual disability	Congenital leucopenia, neutropenia, B and T cell lymphopenia
Hoyeraal-Hreidarsson syndrome (HHS)	*DKC1*	Microcephaly, cerebellar hypoplasia, IUGR	Bone marrow failure, CID or $T^+B^-NK^-$ SCID
Folate and cobalamin metabolism defect	*PCFT, TCN2, MTHFD1*	Failure to thrive, weakness, mental retardation, megaloblastic anemia, neurological disease	Pancytopenia, SCID-like phenotype, hypogam-maglobulinemia
Anhydrotic ectodermic dysplasia with immunodeficiency	*NEMO*	Hypohidrosis, hypodontia, conical teeth, facial dysmorphism	SCID/CID-like phenotype

IUGR, intrauterine growth restriction.

ear anomalies/deafness, may suffer from a $T^-B^+NK^+$ SCID and, in some cases, OS.[103] The disorder is caused by mutations in the chromodomain helicase DNA binding protein 7 gene (*CHD7*), a member of the chromo domain helicase DNA binding domain family of adenosine-5′-triphosphate dependent chromatin remodeling enzymes. CHD7 is expressed throughout the neural crest containing mesenchyme of the pharyngeal arches, suggesting a pathogenetic overlap between CHARGE and DGS.

In other syndromes, several peculiar skeletric abnormalities are the main feature, which lead the patient to the medical attention, as observed in patients with cartilage–hair hypoplasia (CHH), characterized by severe disproportionate short stature due to short limb with metaphyseal dysostosis, sparse hair and neural dysplasia of the intestine,[104] or in Schimke immuno-osseous dysplasia, which sometimes may show a CID phenotype.

In humans, defects in gene involved in telomere maintenance (*TERT*, *TERC*, *DKC1*, *WRAP53/TCAB1*, *NOP10*, *NHP2*, and *TINF2*) are responsible for the dyskeratosis congenita (DC), a rare congenital disorder characterized by progressive bone marrow failure, premature aging, mucocutaneous abnormalities, and cancer predisposition.[106] The most sever infantile variant of X-linked DC is the Hoyeraal-Hreidarsson syndrome (HHS), whose main clinical features are microcephaly, cerebellar hypoplasia, and intrauterine growth retardation. The early-onset bone marrow failure usually leads to either a combined immunodeficiency or a $T^+B^-NK^-$ SCID, which may require HSCT.[107]

Recently, several inborn errors in folate and cobalamin metabolism have been described as having a profound impact on many systems, including hematopoiesis and neuronal function. Immunodeficiency of variable degrees has been associated with defects in these pathways. A CID phenotype

characterized by lymphopenia, responsiveness to folate replacement therapy, and severe bacterial and viral infections has been described in patients with functional methionine synthase deficiency caused by hereditary folate malabsorption due to deficiency in the proton coupled folate transporter (PCFT) and in transcobalamin II (TCN2); this CID usually presents in early infancy in untreated patients as failure to thrive, weakness, pancytopenia, and intellectual disability. Recently, exomic sequencing demonstrated that heterozygous mutations in the trifunctional protein MTHFD1 is responsible for a SCID-like phenotype characterized by $T^-B^-NK^-$ lymphopenia, marked hypogammaglobulinemia, megaloblastic anemia, and neurologic disease.[108] A partial immune reconstitution after vitamin B12 and folate replacement therapy has been documented.

In summary, it must be noted that several syndromes, together with the more typical severe manifestations, can share clinical and immunological signs of SCID/CID, as for example patients affected by NEMO deficiency.

Recently identified combined immunodeficiencies

Combined immunodeficiency (CID) is a group of genetic heterogeneous disorders characterized by severe recurrent infections, moderate reduction of T and B lymphocytes, and impaired cellular and humoral functionality that may reflect late defects in T cell development and function.[109,110] In most cases, it is not always easy to distinguish between patients affected with more severe forms and those with CID. Furthermore, a greater difficulty in making a clear classification is due to the fact that many inborn defects, which underlie these immune disorders, have recently been associated with both SCID and CID, in particular hypomorphic mutations. Several genetic defects responsible for a wide number of clinical conditions are comprised in this group (Table 2).[111] Besides the well-known genetic defects responsible for MHC class I (*TAP1*, *TAP2*, *TAPBP*) or class II deficiency (*CIITA*, *RRFX5*, *RFXAP*, *RFXANK*) associated with a predominant $CD8^+$ or $CD4^+$ selective deficiency respectively, the very rare *CD8A* defects and many others (see the new International Union of Immunological Societies classification, Ref. 111) have been identified recently. Since the number of these conditions is large, we have chosen to discuss only the most common form associated with new phenotypes and novel ones reported over the past 3 to 4 years.

ZAP70-related immunodeficiency is inherited in an autosomal recessive manner. It is caused by abnormal TCR signaling, which leads to a selective absence of $CD8^+$ T cells and normal or elevated numbers of non-functional $CD4^+$ T cells. ZAP70 has a key role in both mature T cell signaling and differentiation of thymic precursors. Finally, in some patients peculiar phenotypes have been observed. In particular, some patients exhibit an attenuated phenotype with a late onset disease and preserved production of $CD4^+$ T follicular helper (T_{FH}), T helper type I (T_H1), T_H17, and T_{reg} cells. Immune dysregulation and severe erythroderma resembling OS have also been described, characterized by skin infiltrative lesions with activated $CD4^+$ T cells in the peripheral blood.[113]

Thanks to next generation sequencing technologies, which have provided a powerful tool to identify the molecular cause of PIDs of unknown genetic origin, new defects have been detected, even though in most cases the genetic cause still remains unknown.

Whole-exome sequencing recently demonstrated the presence of deleterious mutations in the phosphoglucomutase 3 gene (*PGM3*) in three unrelated subjects with recurrent infections, congenital leukopenia, neutropenia, B and T cell lymphopenia, and progression to bone marrow failure due to a congenital disorder of glycosylation (CDG). Two of the three children also had skeletal anomalies characterized by short stature, brachydactyly, dysmorphic facial features, and intellectual disability.[105] Thanks to this technology, Kotlarz *et al.* identified in 2013 two distinct homozygous loss of functions mutations in the interleukin-21 receptor gene (*IL21R*) in two unrelated children affected with cryptosporidiosis, chronic cholangitis and liver disease, recurrent upper and lower airway infections, and failure to thrive.[99] IL-21R binds to common γc and signals via JAK/STAT pathways.[100,101] The authors observed that the mutation was responsible for the aberrant trafficking of the IL-21R to the plasma membrane and for the abrogation of IL-21 ligand binding. These molecular alterations lead to defective phosphorylation of STAT1, STAT3, and STAT5. The immunophenotype of these patients was normal, but abnormal proliferation induced by

IL-21 and defects in immunoglobulin class-switching in B cells and NK cell cytotoxicity were documented. A defect in T cell secretion of several cytokines, including T_H17-associated cytokines IL-17F and IL-22, was reported, thus putatively explaining the increased susceptibility to cryptosporidial infection in these patients.

In the last few years mutations in the CARD9–BCL10–MALT1 (CBM) complex involved in NF-κB signaling have been associated with PID. In particular, autosomal recessive mutations in MALT1 gene have been described in patients with CID and severe bacterial, fungal and viral infections.[114] The MALT1-deficient T cells are not able to degrade IκBα or produce IL-2 following T cell activation. BCL10 has a role in several immune pathways critical for the function of the innate and the adaptive immune systems, and for the response to bacterial and fungal infections. Mutations in *BCL10* and other genes encoding for proteins interacting with MALT1, such as *CARD11* and *CARD9*, have also been recently described. Patients with BCL10 deficiency show a profound defect of memory T and B cells and severe hypogammaglobulinemia, with a reduction of CD69 and CD25 percentages and ICOS levels.[115] Even though CARD9 deficiency has been shown to selectively compromise defenses toward a limited number of fungal infections, mutations in CARD11, which plays a crucial role in the differentiation of both neuronal and immunologic tissues as a scaffold protein, are associated with a more profound CID characterized by abnormal T cell proliferation to anti-CD3/CD28 stimulation, expansion of late transitional B cells, mature B cells deficiency, and hypogammaglobulinemia.[101] Furthermore, CARD11-deficient T cells do not produce normal amounts of IL-2 or upregulate the IL-2 receptor α chain (CD25) after TCR stimulation, which contributes to T_{reg} cell deficiency in these patients.

Mutations in tetratricopeptide repeat domain 7A (TTC7A), a member of the large family of proteins containing the tetratricopeptide repeat (TPR) domain, have recently been found in patients affected with CID and multiple intestinal atresia (MIA).[116] MIA is a clinical condition that can be isolated or may occur in association with variable grades of immunodeficiency ranging from SCID to a mild decrease of T cells and partially preserved thymic function. However, in all these genetic forms, profound $CD8^+$ T cell lymphopenia, reflecting the impaired cellular immunity and the defective thymopoiesis, has been observed. Severe hypogammaglobulinemia is also frequent. A higher frequency of bloodstream infections due to intestinal microbes has also been reported.

The clinical and immunological phenotypes of Ras homolog family member H gene (*RHOH*) deficiency is characterized by naive $CD4^+$ T cell deficiency, absence of recent thymic emigrants, increased number of effector memory T cells, restricted T cell repertoire, and reduced *in vitro* proliferation via CD3 stimulation.[117] Expressed mainly in hematopoietic cells, RhoH is a small GTPase that mediates interaction between Zap70 and LCK. RhoH deficiency determines both alterations in pre-TCR–mediated signaling and in positive selection, as observed in Zap70 deficiency. Expansion of memory T cells has also been observed in other CIDs, such as deficiency of DOCK8 or MST1. DOCK8 deficiency is an autosomal recessive form of CID associated with a hyper-IgE phenotype. Viral infections (especially of the skin) and malignancies are very common. Lymphopenia of $CD4^+$ and $CD8^+$ T cells, or predominantly $CD4^+$ lymphocytes, may be found. In addition, DOCK8 deficient patients exhibit defective differentiation of T_H17 cells and a reduction of B lymphocytes.[118]

The lymphocyte specific kinase LCK is involved in the initiation of signaling from the TCR [121] through the adaptor protein unc-119 lipid binding chaperone (UNC119). Recently, mutations in LCK or UNC119, which impairs LCK activation and signaling, have been identified. Main features of this phenotype include $CD4^+$ T cell lymphopenia, a restricted T cell repertoire, and impaired TCR signaling.[122] Patients with LCK deficiency frequently present with immune dysregulation and autoimmunity. Mutations in the magnesium transporter protein1 gene (*MAGT1*) result in a CID phenotype characterized by $CD4^+$ lymphopenia and abnormal T cell proliferation, which are responsible for chronic viral infections and EBV-related lymphoma, respectively.[123] Recently, a CID was observed in four unrelated patients with mutation of inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (*IKBKB*); the patients had severe bacterial, viral, fungal, mycobacterial infections associated with failure to thrive and neurological impairment. The immunological phenotype was

characterized by a/hypogammaglobulinemia and absence of T_{reg} and γ/δ T cells. Even though T cell counts were normal, all the patients exclusively showed naive T and B lymphocytes.[124]

Newborn screening for SCID

Recently, T cell receptor excision circles (TREC)–based newborn screening has been implemented in several countries. Compared with patients identified by the clinical features, patients identified through newborn screening programs, similar to children identified because of a positive familial history, can receive an early and accurate diagnosis by one month of life and then undergo HSCT or gene therapy by 3 months of age, before the occurrence of severe complications. This results in a significantly improved outcome.[125,126] The TREC assay, based on the detection of intracellular accumulation of products derived from process of T cell receptor gene splicing and rearrangement, is able to detect several defects, which result in either SCID or profound T cell lymphopenia that is also seen in patients affected with 22q11.2DS, CHH, CHARGE, and AT.[127] However, one limitation of the TREC assay is that it is not able to identify all forms of CID or atypical SCID. Some genetic disorders, such as deficiency of ZAP70, late onset ADA, Nijmegen breakage syndrome, MHC class II deficiency, and many others, are likely to be missed because TRECs are usually found at normal levels. The identification of kappa-deleting recombination excision circles (KREC), a sensitive marker of newly formed B cells, increases the possibility of identifying other forms of SCID/CID that are associated with low numbers of B lymphocytes, such as NBS and late onset ADA. Furthermore, it has been reported that tandem mass spectrometry can easily identify abnormal purine metabolites in newborns with typical or late onset ADA and PNP deficiency,[19] thus increasing the spectrum of disorders detectable through newborn screening.

Conclusions

SCIDs are a heterogeneous group of syndromes related to alterations of distinct genes that cause abnormalities in the maturation and/or function of T, B, and/or NK cells. Recently, advances in next generation DNA sequencing have allowed new gene identification through whole exome sequencing or whole genome sequencing of several forms of SCID and CID of unknown cause. The phenotypic and the molecular heterogeneity of SCIDs, as revealed by the expanding phenotypes observed, is making traditional classification of this group of disorders very intricate. Frequently, different mutations in the same gene can lead to different clinical phenotypes, such as OS, leaky SCID, or CID, that may even be inherited with different mechanisms.

In this review we have focused in detail on different forms of SCID and CID, paying attention to the distinctive peculiar clinical and laboratory features, in order to provide information to clinicians for recognizing and carefully managing these novel forms of PIDs.

Conflicts of interest

The authors declare no conflicts of interest.

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2.2 Common γ chain over-expression in B-precursor acute lymphoblastic leukemia primary cells from pediatric patients: implications in leukemia cell cycle progression and survival

The common γ-chain (γc) is a shared component of receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines play a critical role in development, proliferation, survival and differentiation of cell types of both the innate and adaptive immune systems (55, 56).

Alterations of the IL-2RG gene, that encodes for γc, leads to X-linked Severe Combined Immunodeficiency (X-SCID), a disorder characterized by the absence of T and natural killer cell and a normal number of non- functional B cells (57, 58). This disorder is fatal in the first months of life without therapy and for which the bone marrow transplantation represents the conventional successful therapy.

Recently, new therapeutic approaches, such as gene therapy, have been considered for the immunological reconstitution. In 2 distinct gene therapy trials for X-SCID, immunological reconstitution has been documented in 17 out of 20 patients. Unfortunately, 5 of these patients developed leukemia and, only in 2 of them an insertional mutagenesis in LMO2 oncogene was documented (59, 60). Moreover, it was reported in mice that IL2RG cooperates with LMO2 in inducing hematopoietic tumors (61). Of note, no clonal lymphoproliferation has been reported, in patients receiving gene therapy for ADA deficiency, despite the observation of a similar frequency of integration sites near LMO2 and other proto-oncogenes (62, 63). Moreover, overexpression of γc, in X-SCID mice, led to T-cell lymphomas and thymic hyperplasia in a third of cases, and no common integration site was found in these mice (64). These results suggested that insertional mutagenesis was not the only cause of leukemogenesis, raising the possibility that IL-2RG is oncogenic per

se. In addition, it is noteworthy, that genes involved in normal cellular processes, including signal transduction, cell cycle control, DNA repair, cell growth and differentiation, such as growth factors and their receptors, transcriptional regulation, senescence and apoptosis, have been implicated in cancer.

By taking advantage from these evidences, we hypothesize that γc could be potentially involved in leukemogenesis. In keeping with this, our previous study we documented an over-expression of γc protein in continuous malignant hematopoietic cell lines, and a direct correlation between its expression and spontaneous cell growth.In addition, an increased expression of all D-type cyclins and a direct correlation between the amount of γc and cyclins A2 and B1 expression was noted, thus implying a critical regulatory role of γc on cell proliferation of continuous malignant hematopoietic cells (65).

Of note, it is well known that ILs using γc-receptorsplay a role either in T-cell development and function, under physiological circumstances, and in enhancing cell growth of T-cell Acute Lymphoblastic Leukemia (T-ALL) cells (66, 67).

In order to evaluate whether γc molecule is selectively involved in the biology of specific types of leukemia, and not only in T-ALL, in this study we analyzed the γc expression profile and γc-signaling in different types of leukemia. We evaluated the γc expression in γc primary cells of Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), B-precursor Acute Lymphoblastic Leukemia (B-pre ALL), and T-ALL. We also investigated the potential mechanisms by which γc exerts its role in leukemia cell proliferation and survival.

In particular, we evaluated the expression levels of IL2RG mRNA in cells derived from bone marrow aspirates of 39 newly diagnosed patients affected with CML, AML, B-preALL and T-ALL, and healthy controls, through Real-Time PCR.

We found that IL2Rg mRNA expression was increased in leukemic cells as compared to healthy control (mean ± SE =1,74 ± 0,21) (Figure 7). In particular, this overexpression was found in lymphoblastic leaukemia cells (mean ± SE =2,62 ± 0,4) (Figure 7).

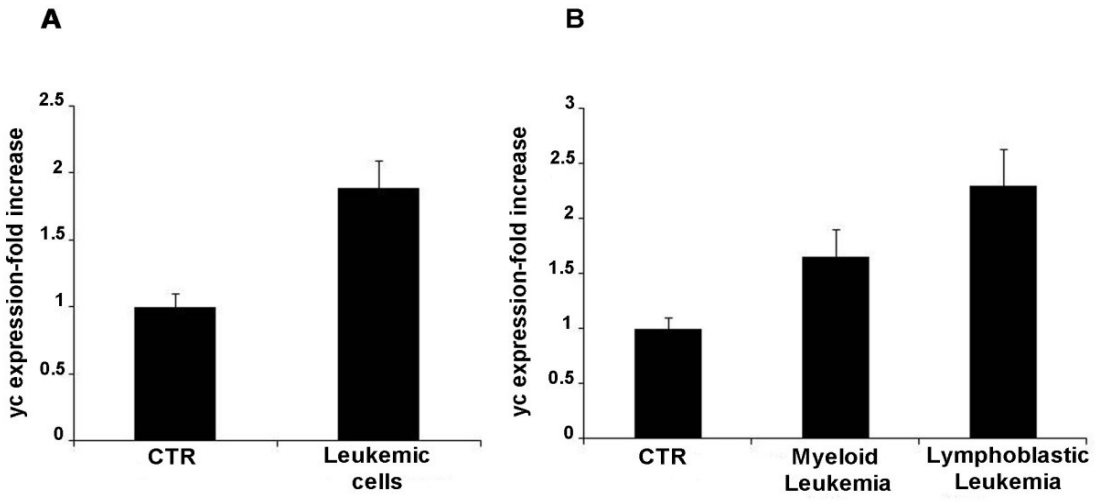

Figure 7. IL2RG mRNA expression was increased in leukemia cells. **A-B,** Total RNA from leukemic cells and controls was extracted and reverse transcribed. The expression levels of *IL2GR* were analyzed by qRT-PCR in leukemia cells (n=39) from AML (n=15), CML (n=10), ALL (n=14) patients and controls. *IL2GR* mRNA amount was mainly increased in ALL cells. The relative gene expression is a ratio *IL2RG*/β-actin fold change. Each histogram represents the mean ± SE.

γc-signaling cytokines play a central role in T-cell development and in the mitogenic potential of primary T-ALL cells (66, 67), suggesting the implication of γc-signaling in this leukemia subtype. Differently from what expected, we found a higher expression of *IL2RG*mRNA in B-pre ALL (mean ± SE = 2.55 ± 0.4) (*P< 0.05*) than in T-ALL cells (mean ± SE = 1.57 ± 0.3) (Figure 8).

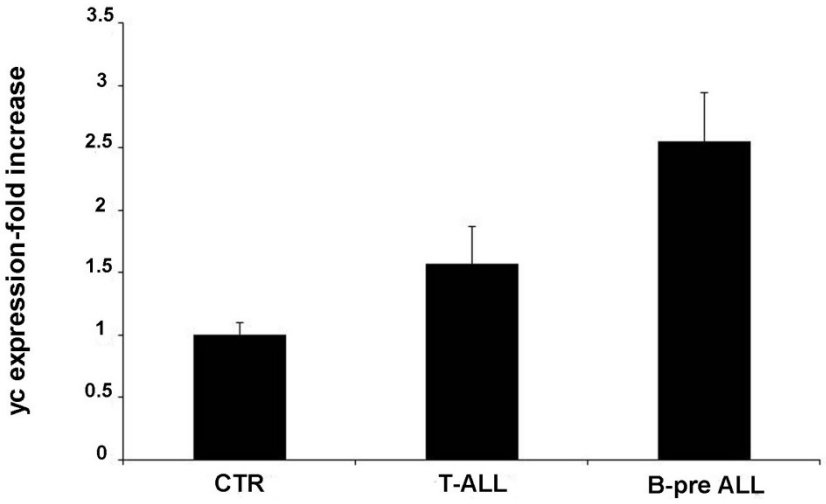

Figure 8. IL2RG mRNA expression was higher in B-pre ALL than in T-ALL cells. The expression levels of IL2RG were analyzed by qRT-PCR in B-pre ALL (n=10) and T-ALL (n=4) cells. The relative gene expression is a ratio IL2RG/β-actin fold change. Each histogram represents the mean ± SE.

The master regulator proteins of cell cycle progression are the cyclin-cdk complexes, an evolutionarily conserved family of proline-dependent serine/threonine kinases (68).

In ALL abnormal expression and function of cell cycle proteins, in particular of D-type cyclins, has already been documented in B-cell malignancies (69). These cyclins are under the control of PI3-AKT signaling pathway, which is regulated by γc. Thus, we investigated expression pattern of *D-type cyclins* through qRT-PCR in B-pre ALL cells.

The results showed that D-type cyclins more were expressed in B-pre ALL cells than healthy controls (Figure 9). In particular, all the three cyclins were over-expressed in the B-pre ALL (*D1 cyclin*: mean ± SE = 21.90 ± 2.8; *D2 cyclin*: mean ± SE = 5.18 ± 0.80; *D3 cyclin*: mean ± SE = 3.22 ± 0.61) ($P<0.05$ for *D1 cyclin* expression vs controls) (Figure 9).

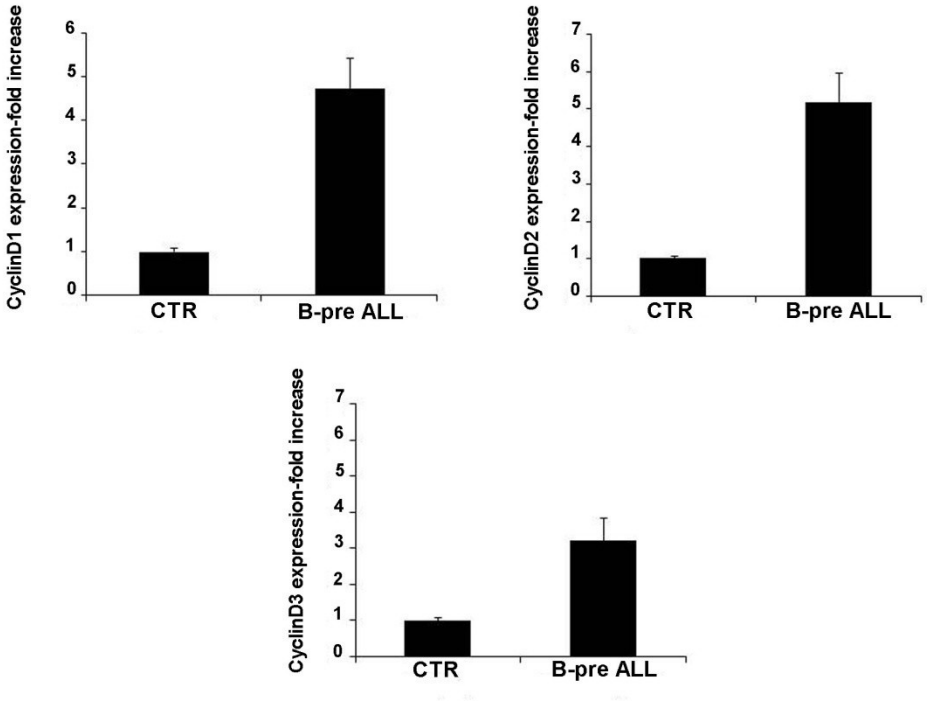

Figure 9. D1, D2 and D3 cyclins expression levels were increased in B-pre ALL cells. Analysis of D-type cyclins expression using qRT-PCR revealed up-regulation of all D-type cyclins. The mRNA levels were expressed relative to β-actin expression levels. Each histogram represents the mean ± SE.

A direct correlation between *IL2RG* and *cyclins D2* (R = 0.82) and *D3* (R = 0.76) expression was found, while no correlation between γc expression and *cyclin D1* was observed (Figure 10).

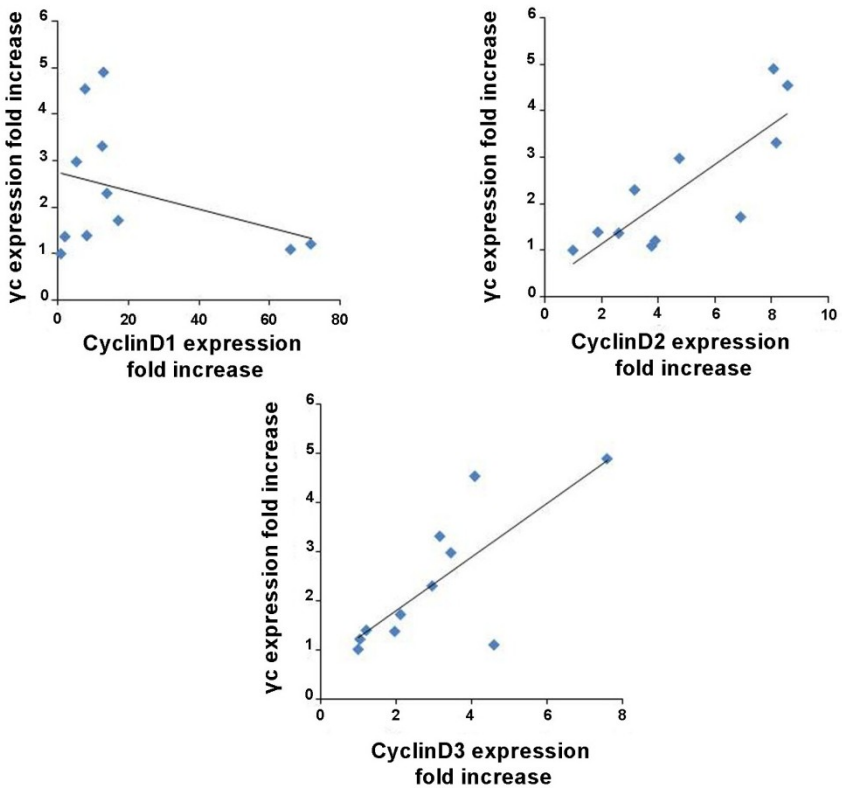

Figure 10. Positive relationship between *IL2RG* and *cyclins* D2 and D3 expression levels. A direct positive correlation between *IL2R*G and *cyclins* D2 and D3 was found, as demonstrated by Pearson correlation coefficient (cyclin D2: $R = 0.82$; cyclin D3: $R = 0.76$). No correlation between *IL2RG* and cyclin D1 was found.

Cancer development and progression mainly relies on the ability of tumor cells to escape apoptosis more than their normal counterpart (70). BCL-2 family members, including BCL-2, BCL-XL, BAX, BAD, are involved in the regulation of apoptosis (71), and an abnormal expression or function of these proteins has been well documented in human cancer (72). Noteworthy, through the activation of PI3-K/AKT signaling pathway, γc also regulates genes involved in this biological process (73).

We evaluated the expression level of the anti-apoptotic *BCL-XL* by quantitative qRT-PCR in order to investigate the mechanisms by which γc promotes cell survival in B-pre ALL. We observed that in B-pre ALL cells, *BCL-XL* mRNA

was 57% than controls (mean ± SE=0.57±0.12) (Figure 11), thus suggesting the presence of alternative mechanisms of the pro-survival effect of γc.

Recent evidence suggests that autophagy favors cell survival in the tumor microenvironment (74). BCL-2 family proteins are involved in autophagy through the inhibition of BECLIN-1, an important mediator of autophagy.

Therefore, by quantitative qRT-PCR, we observed that the expression level of *BECLIN-1* mRNA was increased (mean ± SE= 4.73 ± 0.71) (*P<0.05*) in B-pre ALL as compared with the controls (Figure 11).

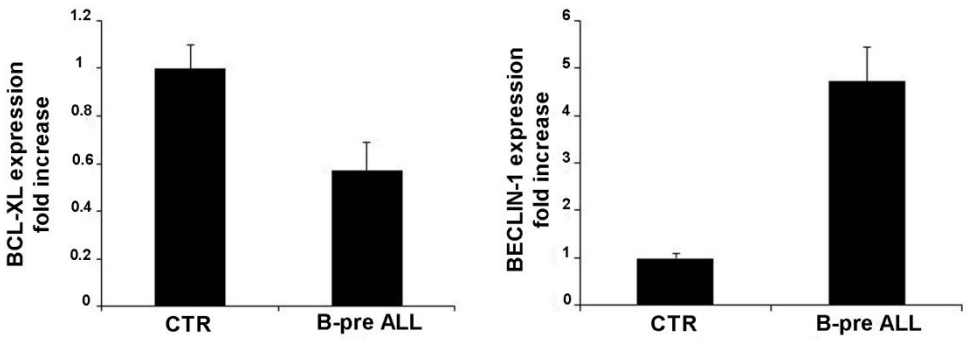

Figure 11. γc promotes leukemia cell survival *via* autophagy through the up-regulation of *BECLIN-1*. *BCL-XL* and *BECLIN-1* mRNA expression was analyzed through qRT-PCR. *BCL-XL* mRNA was 57% of controls. *BECLIN-1* expression was greatly increased. The mRNA levels were expressed relative to β-actin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

To confirm increased BECLIN-1 expression, we performed western blot analysis on total cell lysates obtained from B-pre ALL patients. Our results demonstrated that amount of BECLIN-1 is increased in B-pre ALL cells as compared with control (Figure 12).

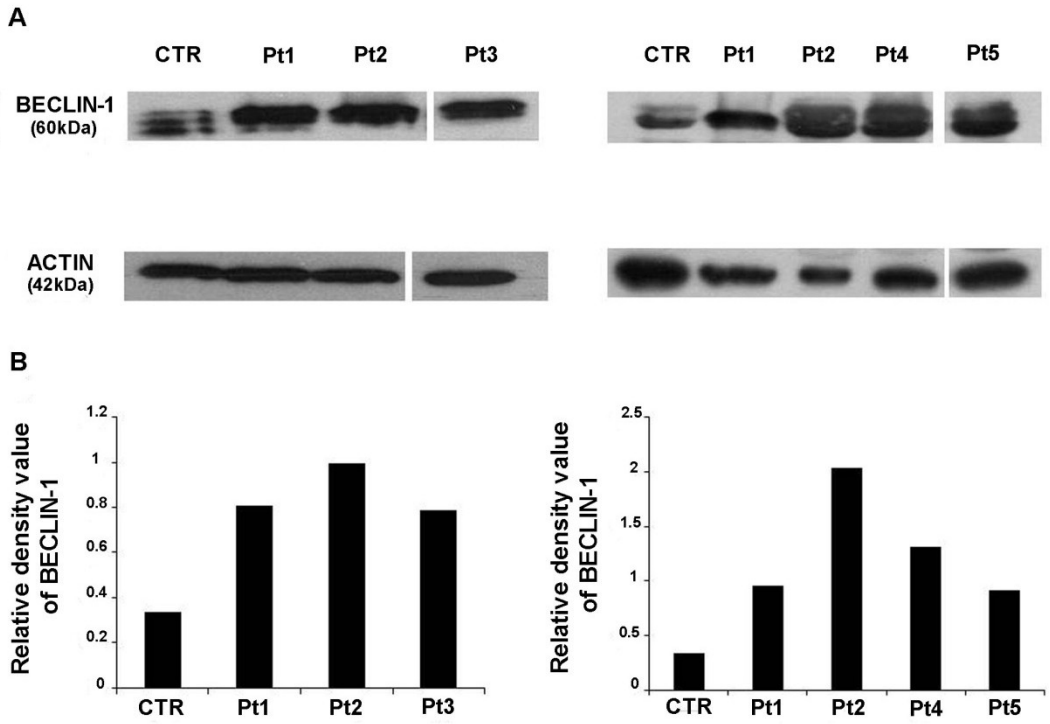

Figure 12. Western blot confirmed BECLIN-1 up-regulation in B-pre ALL cells. **A,**Whole cell extracts were prepared from B-pre ALL and control cells. BECLIN-1 expression was determined by western blotting. The figure represents two different experiments. **B,**Densitometric analysis of the western blotting was performed by using ImageJ program. All data were normalized to β-actin.

In this study, we documented an up-regulation of *IL2RG* in several primary leukemia cells, and, for the first time, in B-pre ALL cells. This finding is unexpected, in that it is well established that the γc-signaling pathway under physiological circumstances plays a role in the T-cell development.

The regulatory role of a number of cytokines, as interleukins and colony-stimulating factors, on the survival, growth, differentiation and apoptosis of leukemic cells has been extensively documented both *in vitro* and *in vivo* (75, 76). ILs using γc-receptors,along with the role in T-cell function and development, are enhancers of cell growth of T-ALL cells (66, 67). Previous studies, through knock-out strategy or neutralization of γc-dependent cytokine receptors, have already shown a potential

clinical implication of the modulation of γc-dependent signaling pathways in cancer treatment (56).

As for the molecular mechanisms by which γc promotes cell cycle progression in B-pre ALL, we investigated D-type *cyclins*, which are important regulators of the passage through the early G1 and late G1 phase of cell cycle, a critical check-point that irreversibly directs the cells to undergo one cell division (68, 77).

We found that D-type *cyclins* mRNA expression is increased, with a prominent up-regulation of *cyclin D1*. The overexpresssion of D-type *cyclins* has been previously reported in B-cell malignancies (69, 78-80). Moreover, alterations of *cyclins D1*, *D2* and *D3* have also been documented in ALL (81). The prominent increase in our study of *cyclin D1* mRNA in B-pre ALL is in keeping with what previously observed in B-cell neoplasias (69, 82), where it was overexpressed, differently from normal lymphoid cells, where this *cyclin* is absent (82). By contrast, *cyclins D2* and *D3* play a role in hematopoiesis also under physiological conditions. The *cyclin D2-/-* murine model exhibits an impairment of B-lymphocyte proliferation (83, 84). *Cyclin D3*$^{-/-}$ mice are affected with severe defects in B- and T-lymphocyte development and neutrophil maturation (81).

In our study, a positive correlation between the amount of γc and the expression of *cyclins D2* and *D3* has also been found. This would indicate that the activation of these *cyclins* and *γc* is under an unique regulatory control in B-pre ALL, thus being implicated in the pathogenesis of the lymphoproliferation.

Due to the role of apoptosis in cancer development (70), we evaluated in B-pre ALL cells the expression levels of the anti-apoptotic *BCL-XL* molecule, a member of the BCL-2 family proteins. This molecule plays a pivotal role in

promoting tumor cell survival (70, 85). In addition, a deregulation of Bcl-2 proteins expression has been documented in several types of human cancer (72, 86). We found a down-regulation of *BCL-XL* expression, thus implying the presence of alternative, or additional, mechanisms capable to escape cell death through the over-expression of *γc* in B-pre ALL cells.

The high proliferation rate of cancer cells requires continuous source of energy and nutrients, but the tumor microenvironment is not able to supply these essential needs for cancer cell survival (87). Several studies highlighted the critical role of autophagy in protecting cells against a shortage of nutrients, through removal and recycling of damaged molecules and organelles, even though the exact molecular and biochemical mechanism by which cancer cells obtain energy sources under these limiting conditions, remains to be elucidated (88, 89). In our study, we found in B-pre ALL cells increased *BECLIN-1* mRNA levels and protein amount, as compared with controls. Beclin-1 is a component of the class III phosphatidylinositol-3-kinase (PI(3)K) complex, that participates in autophagosome nucleation (90). The molecule is activated by a number of proteins, including AMBRA1, UVRAG and Bif-1, (91, 92), and, in turn,promotes the activation of the PI(3)K protein (Vps34) and the formation of autophagosomes. Its autophagy-promoting ability is suppressed by the antiapoptotic Bcl-2 family members through the direct binding of BH3-domain-only (90).

Evidence indicates that the predominant role of autophagy in cancer cells is to confer stress tolerance, which allows tumor cell survival (93). In keeping with this, knockdown of essential autophagy genes in tumor cells re-builds or potentiates the activation of cell death (94). By contrast, if microenvironment stress persists, excessive autophagy may ultimately lead to autophagic cell death (95).

Thus, in our study, the prominent up-regulation of *BECLIN-1* expression in B-pre ALL cells, contextual with the decreased expression of *BCL-XL*, is in favor of a role of autophagy activation in the pro-survival effect of γc, by promoting autophagosome nucleation in the absence of the inhibitory effect exerted by BCL-XL on BECLIN-1.

The data here described document for the first time a prominent up-regulation of *IL2RG* expression in B-pre ALL cells, suggesting a potential selective role of γc in this leukemia. Importantly, we described the potential mechanisms by which γc may promote proliferation and survival in B-pre ALL cells. We demonstrated a direct correlation between mRNA levels of *IL2RG* and *cyclins D2* and *D3* in B-pre ALL cells, thus implying a role of γc in promoting cell cycle progression by transcriptional regulation of these genes. Moreover, we observed BECLIN-1 increased expression in B-pre ALL cells, suggesting the activation of autophagy, as a potential mechanism by which γc promotes cell survival.

Our data imply a role of γc in the pathophysiology in B-pre ALL and further studies in this field will contribute to develop innovative therapeutic strategies in this leukemia.

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

CHAPTER III

The role of the epithelial cells in the immune system

The thymus is the primary lymphoid organ that supports T the development of fully mature and self-tolerant T cells (96). This organ is organized in two lobes, each one divided in two main regions: a cortical area, a medullary and a transitional area, named cortico-medullary junction (CMJ), characterized by abundant blood vessels (97). These regions provide appropriate cellular interactions, cytokines and chemokines required to induce a full thymocyte development, a process intimately linked to the specialized functions of thymic stromal cells (TSCs) and to the thymus architecture (98). An important feature of the thymic microenvironment relies on its three-dimensional (3D) organization, consisting of an ordered architecture of TSCs, which may be epithelial or mesenchymal in origin, through which the developing thymocytes migrate and mature. This 3D configuration maximizes the interaction of developing thymocytes with the supporting stromal cells, allowing a proper intercellular cross-talk, integral to the development of either T cells and TSCs (99).

The intrathymic T-cell development consists of several processes that require the dynamic relocation of developing lymphocytes within multiple compartments of the thymus (97). The developmental pathway is divided into three subsequent steps, charachterized by the expression of specific immunophenotypic patterns: the $CD4^{-}CD8^{-}$ double negative (DN) stage, the $CD4^{+}CD8^{+}$double positive (DP) stage, and the $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ single positive (SP) stage (Figure 13).

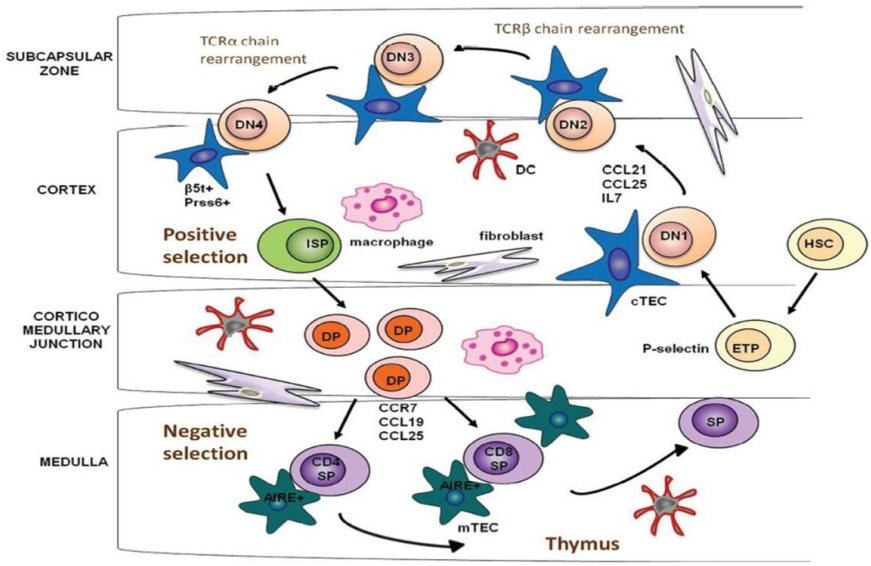

Figure 13. T-cell development and lympho-stromal crosstalk (97).

From the early T-cell lineage progenitor (ETP) stage to the double-negative 3 (DN3) stage, the T-cell differentiation is independent from the T cell receptor (TCR), but is strictly dependent on the migration through the distinct thymic structures (100)] and on the expression levels of specific transcription factors.

When TCR interacts with low-avidity with the peptide-MHC ligands, DP thymocytes receive survival signals. This process, referred to as positive selection, allows "productive" T cells to potentially react to foreign antigens, but not to self-antigens. Within the thymus, DP thymocytes are ready to differentiate into single positive (SP) cells, CD4+CD8- or CD4-CD8+, that relocate to medulla. At this site, newly generate SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator- (AIRE-) expressing mTECs (101). The cells which are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity.

A remarkable number of similarities are shared between the epithelial and stromal cells of the thymus and keratinocytes and fibroblasts of the skin. Both thymic and skin epithelial cells selectively express the FOXN1 transcription factor, which

plays a critical role in differentiation and survival of these specialized cells (97). Hassall corpuscles, a product of medullary thymic epithelial cells, contain keratins identical to those in the stratum corneum of the skin. In addition, keratinocytes express many keratins expressed in TECs. Moreover, the Notch pathway is shared between thymic stroma and skin elements, playing an important role in regulating epidermal differentiation, early stage thymocyte maturation and T-cell lineage commitment (97). By contrast, a major difference between thymus and skin is the architecture of each organ, in that the skin epithelial cells are mostly distributed along a basement membrane, differently from epithelial cells of the thymus, which are organized in a 3D configuration.

3.1 Unraveling the link between ectodermal disorders and primary immunodeficiencies

Primary immunodeficiency disorders (PIDs) represent a heterogeneous group of inherited disorders characterized by poor or absent function in one or more components of the immune system, that result in chronic, recurrent and life-threatning infections if not promptly diagnosed and treated (2).

Recent evidence highlights that the skin participates in a host defenses either acting as a primary boundary for germs, as the principal site of environment–host interactions, or directly in the developmental process of the immune system. As a matter of fact, skin and skin annexa abnormalities, such as skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, can be not infrequently associated with distinct forms of immunodeficiency and may be a warning sign of immunodeficiency, since both epidermal and thymic epithelium have ectodermal origin.

Several forms of PIDs are characterized by developmental abnormalities of the skin and immunodeficiency. Elevated IgE levels represent the immunological hallmark of hyper-IgE syndrome, characterized by severe eczema and susceptibility to infections (102). Ectodermal dysplasia (ED) is a group of rare disorders that affect tissues of ectodermal origin. Hypoidrotic ED (HED), the most common form, is inherited as autosomal dominant, autosomal recessive or X-linked trait (XLHED). HED and XLHED are caused by mutations in *NEMO* and *EDA-1* genes, respectively, and show similarities in the cutaneous involvement but differences in the susceptibility to infections and immunological phenotype (103, 104).

Alterations in the transcription factor *FOXN1* gene, expressed in the mature thymic and skin epithelia, are responsible for human and murine athymia and prevent

the development of the T-cell compartment associated to ectodermal abnormalities such as alopecia and nail dystrophy (105).

The association between developmental abnormalities of the skin and immunodeficiencies suggest a role of the skin as a primary lymphoid organ. Recently, it has been demonstrated that a co-culture of human skin-derived keratinocytes and fibroblasts, in the absence of thymic components, can support the survival of human haematopoietic stem cells and their differentiation into T-lineage committed cells (97).

In the last decades, the field of PID has been deeply studied, eventually leading to an overall better knowledge and nosografic re-classification of the different forms so far identified. In particular, several novel forms have been described unraveling new clinical and genetic aspects. Nevertheless, it has been documented that an inappropriate or late diagnosis of PID by clinicians often occurs, thus indicating the strong need of an update on the novel clinical associations of different forms and alarm signals.

An overview on this topic and on the most common immune disorders associated with ectodermal alterations have been published as Reviews on *International Reviews of Immunology,* for the manuscript see below.

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ARTICLE

Unraveling the Link Between Ectodermal Disorders and Primary Immunodeficiencies

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Primary immunodeficiencies (PIDs) include a heterogeneous group of mostly monogenic diseases characterized by functional/developmental alterations of the immune system. Skin and skin annexa abnormalities may be a warning sign of immunodeficiency, since both epidermal and thymic epithelium have ectodermal origin. In this review, we will focus on the most common immune disorders associated with ectodermal alterations. Elevated IgE levels represent the immunological hallmark of hyper-IgE syndrome, characterized by severe eczema and susceptibility to infections. Ectodermal dysplasia (ED) is a group of rare disorders that affect tissues of ectodermal origin. Hypoidrotic ED (HED), the most common form, is inherited as autosomal dominant, autosomal recessive or X-linked trait (XLHED). HED and XLHED are caused by mutations in *NEMO* and *EDA-1* genes, respectively, and show similarities in the cutaneous involvement but differences in the susceptibility to infections and immunological phenotype. Alterations in the transcription factor *FOXN1* gene, expressed in the mature thymic and skin epithelia, are responsible for human and murine athymia and prevent the development of the T-cell compartment associated to ectodermal abnormalities such as alopecia and nail dystrophy. The association between developmental abnormalities of the skin and immunodeficiencies suggest a role of the skin as a primary lymphoid organ. Recently, it has been demonstrated that a co-culture of human skin-derived keratinocytes and fibroblasts, in the absence of thymic components, can support the survival of human haematopoietic stem cells and their differentiation into T-lineage committed cells.

Keywords: ectodermal dysplasia, *FOXN1*, hyper-IgE, primary immunodeficiencies, T-cell development

INTRODUCTION

Primary immunodeficiencies (PIDs) include a heterogeneous group of diseases, mostly monogenic, which are characterized by functional/developmental alterations of the immune system. In the last decades, the field of PID has been deeply studied, eventually leading to an overall better knowledge and nosografic re-classification of the different forms so far identified. In particular, several novel forms have been described unraveling new clinical and genetic aspects. Nevertheless, it has been documented that an inappropriate or late diagnosis of PID by clinicians often occurs, thus indicating the strong need of an update on the novel clinical associations of different forms and alarm signals. An overview on this topic would favor an early diagnosis.

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Recent evidence highlights that the skin participates in a host defenses either acting as a primary boundary for germs, as the principal site of environment–host interactions, or directly in the developmental process of the immune system. As a matter of fact, skin and skin annexa abnormalities, such as skin dryness, brittleness of hair, nail abnormalities and abnormal dentition, can be not infrequently associated with distinct forms of immunodeficiency.

In this review, we will focus on the link between skin developmental alterations and PIDs that could help in the early detection of some immunologic disorders.

ECTODERMAL DYSPLASIA AND IMMUNE DEFECTS

Ectodermal dysplasia (ED) is a group of rare inherited disorders that affect two or more tissues of ectodermal origin. Ectodermal dysplasia has an incidence of seven cases per 10,000 live births and to date nearly 200 different forms of ectodermal dysplasia have been described. The main abnormalities involve the skin, which may be dry, thin and hypopigmented, and prone to rashes, eczema or infections. Furthermore, sweat glands may function abnormally, or may not develop at all, and hair is usually hypopigmented, thin and sparse.

The abnormal sweat production may impair body temperature control, thus leading to overheating, especially in hot environments. Airways seromucous glands may also be affected predisposing to respiratory infections because of the absence of the normal protective secretions of the mouth and nose. Defect in meibomian/tarsal glands may lead to dryness of the eye, cataracts, and vision defects. Teeth may be congenitally absent, peg-shaped or pointed. The enamel may also be defective. Typical cranial-facial features include frontal bossing, longer or more pronounced chins and broader noses. Abnormalities in the ear development may cause hearing problems.

Hypoidrotic ED (HED) is the most common form with an incidence of 1:10,000 [1]. This form is inherited as an autosomal dominant (AD), autosomal recessive (AR) or X-linked trait (XLHED). HED derives from mutations in the ectodysplasin-A (EDA) signaling pathway, which leads to the expression of genes implicated in the development of the skin and skin appendage. Mutations in the *EDA* gene on X-chromosome cause approximately 80% of cases of HED (OMIM 305100, XLHED, ectodermal dysplasia, type 1, ED1). A smaller subset of cases is caused by mutations in the EDA receptor (EDAR), the adapter protein (EDARADD), or WNT10A [2, 3], being inherited in an autosomal recessive (ectodermal dysplasia anhidrotic; EDA; OMIM 224900) or autosomal dominant manner (ectodermal dysplasia type 3; ED3; OMIM 129490). EDA regulates organogenesis at multiple levels, from the initiation to the terminal differentiation [4, 5]. The activation of EDA pathway is implicated in the appearance of focal thickenings of the epithelium known as placodes, implicated in the very early stage of skin appendage development. *EDA* gene is a member of the TNF superfamily. EDA ligand binds to the trimeric EDAR receptor, which, in turn upon binding, recruits the EDARADD adaptor via death-domain–death-domain interactions. This cascade, from EDA and EDAR to EDARADD, leads to the activation of the NF-kB pathway. In unstimulated cells, NF-kB is sequestered in the cytoplasm by the inhibitor of the kBproteins (IkB). NF-kB essential modulator (NEMO), also called IkB Kinase (IKK) gamma protein, acts as a regulatory subunit of the IKK complex, comprising two kinase subunits (IKK1/a and IKK2/b), required for the activation of canonical NF-kB pathway. Upon stimuli, IkB is phosphorylated by the IKK, resulting in IkB degradation, NF-kB translocation into the nucleus, and eventually regulation of the target gene expression [6].

Hypomorphic mutations in the NEMO encoded by the *IKBKG/NEMO* gene on the X-chromosome result in HED with immunodeficiency (HED-ID, OMIM 300291) [7–9].

In spite of the similarity of the cutaneous involvement, difference in the susceptibility to infections and in the immunological pattern between HED-ID and XLHED patients is well documented. Due to the pleiotropic role of NEMO, mutations in *IKBKG/NEMO* gene lead to a heterogeneous and severe immunodeficiency. In patients with XLHED, immunologic alterations have never been reported. However, in these patients recurrent bronchial or eye infections have been described and interpreted as a result of reduced bronchial or meibomian/tarsal gland function. The difference between HED-ID and XLHED immune defect may rely on the distinct pathways in which NEMO and EDA-1 are involved. In fact, while both molecules are involved in the ectodysplasin pathway, thus explaining the similarity in the cutaneous involvement, NEMO is also involved in signaling pathways downstream to different receptors, including toll-like (TLRs), interleukin-1 (IL-1Rs), tumor necrosis factor (TNFRs), and B- and T-cell receptors (TCR and BCR). The participation of NEMO in these pathways explains the wider immune defect in HED-ID and its complexity. HED-ID is characterized by unusually severe, recurrent, and sometimes life-threatening bacterial infections of the lower respiratory tract, skin, soft tissues, bones and gastrointestinal apparatus as well as meningitis and septicemia in early childhood. These patients display high susceptibility to infections by Gram-positive bacteria (*S. pneumoniae* and *S. aureus*), followed by Gram-negative bacteria (*Pseudomonas* spp. and *Haemophilus influenzae*) and mycobacteria, as well.

Laboratory features include hypogammaglobulinaemia with low serum IgG (or IgG2) levels, and variable levels of other immunoglobulin isotypes (IgA, IgM and IgE). Elevated serum IgM levels have been described in a number of HED-ID patients with the hyper-IgM6 phenotype [10, 11]. In some patients, a defective ability of B cells to switch in response to CD40 ligand (CD40L) has been described, which may help explain the hyper-IgM phenotype. Defective antibody response to polysaccharide and proteic antigens is the most consistent laboratory feature. Recently, impaired NK activity has also been reported in some [12] but not all [13] patients with EDA-ID. NEMO also acts downstream to TLRs [13–15]. As a consequence, NEMO patients exhibit poor inflammatory response, also due to impaired cellular responses to pro-inflammatory cytokines (IL-1β, IL-18 and TNF-α) [15]. Impaired IL-1β- and IL-18-dependent induction of IFN-γ, impaired cellular responses to IFN-γ-inducible TNF-α, and impaired signaling through TLRS may explain the occurrence of severe mycobacterial disease in these patients.

Different *NEMO* mutations have also been associated with distinct disorders. While loss-of-function mutations cause incontinentia pigmenti (IP), hypomorphic mutations cause two allelic conditions, namely HED-ID and a clinically more severe syndrome, in which osteopetrosis and/or lymphoedema associate with HED-ID (OL-HED-ID; MIM 300301). Mutations in the coding region are associated with the HED-ID phenotype (MIM 300291), while stop codon mutations cause a OL-HED-ID [11, 16–21]. IP (OMIM#308300), which specifically affects females, being lethal in males [22], is caused by a complex rearrangement of *NEMO* gene that results in the deletion of exons 4–10, coding for a shortened protein unable to elicit an NF-κB response. This recurrent rearrangement accounts for 85% of IP patients [16, 17]. Affected females present with Blashko linear skin lesions [23] variably associated with developmental anomalies of teeth, eyes, hair and the central nervous system.

Mutations in other genes involved in NF-κB pathway are responsible for different forms of HED-ID. Gain-of-function mutations of IκBα are able to enhance the inhibitory capacity of IκBα through the prevention of its phosphorylation and degradation, and result in impaired NF-κB activation leading to HED-ID. The developmental, immunologic and infectious phenotypes associated with hypomorphic NEMO and hypermorphic IKBA mutations largely overlap and include EDA, impaired cellular

TABLE 1. Clinical and immunological features of hyper-IgE syndrome (HIES).

Immunodeficiency	Gene	Immunological phenotype	Clinical features	OMIM
HIES-AD	*STAT3*	Reduced Th17 lymphocytes; reduced specific antibody response; reduced switched and no switched B memory lymphocytes	Facial anomalies, eczema, osteoporosis and pathological fractures, teeth anomalies, joint laxity, Stafilococcus aureus infections (pulmonary and cutaneous abscesses, pneumatocele), candidiasis	#147060
HIES-AR	*TYK2*	Altered cytokine signaling	Increased susceptibility to fungi, viruses and intracellular bacteria (mycobacterium, salmonella spp.)	#611521
	DOCK8	Reduced T, B and NK cells, hyper-IgE, reduced IgM levels	Severe atopy, hypereosinophilia, recurrent infections, severe viral and bacterial cutaneous infections, predisposition to cancer	#243700

responses to ligands of TIR (TLR-ligands, IL-1β and IL-18), and TNFR (TNF-α, LTα1/β2 and CD154) super family members leading to severe bacterial diseases.

Recently, mutations in *NF-kB2* gene have been described as responsible for the early onset of common variable immunodeficiency, inherited as an autosomal dominant trait. In the cases so far identified, ectodermal abnormalities, including nail dystrophy and alopecia together with endocrine alterations, have been reported.

PRIMARY IMMUNODEFICIENCIES WITH HYPER-IgE

Elevated IgE levels represent the immunological hallmark of a growing group of PID termed as hyper-IgE syndrome (HIES). The clinical phenotype of these syndromes, along with very high IgE levels (>2000 IU/L), comprises severe eczema and susceptibility to a spectrum of infections, especially staphylococcal and fungal infections, involving the skin and lungs. These disorders can be inherited in an autosomal dominant or autosomal recessive manner (Table 1). Sometimes, sporadic cases have been described. It isn't always easy to differentiate the syndromes from the severe forms of atopic dermatitis, in which high levels of serum IgE, and sometimes viral or bacterial infections, could also occur, since the complete clinical phenotype of HIES often becomes evident only over years. This may cause delay in diagnosis, especially in those patients who have milder forms of the disease.

In 2007, Holland et al. [24] found that hypomorphic mutations of signal transducer and activator of transcription 3 (*STAT3*) gene are responsible for the autosomal dominant form of HIES, characterized by the classic clinical triad represented by recurrent cutaneous "cold" abscesses, recurrent pulmonary infections and increased concentration of serum IgE. Such triad is present in 75% of autosomal dominant cases and in 85% of children with a disease onset before 8 years of age. In many cases, eczema, often with a neonatal onset, is the first sign of the disease.

In the patients with a *STAT3* defect, in addition to Staphylococcus aureus, often methicillin-resistant, infections with other pathogens, such as Haemophilus influenzae and Streptococcus pneumonia, may also be found. Moreover, the recurrent sinopulmonary infections, through the formation of bronchiectasis and some-

times pneumatoceles, represent predisposing factor to colonization by opportunistic agents such as Pseudomonas aeruginosa and Aspergillus fumigatus, with the risk of developing invasive aspergillosis and systemic fatal infections. Another frequent infection is chronic mucocutaneous candidiasis and, to a lesser extent, Pneumocystis jirovecii lung infection. In addition, other fungal pathogens, including Histoplasma, Coccidioides and Cryptococcus, have been reported to cause gastrointestinal infections as well as meningitis in patients with HIES [25]. This increased susceptibility to infections is due to the impairment of Th17-cell function through the alteration of signaling mediated by various cytokines, and in particular IL-6 and IL-22 [26]. Beyond the immunological and infectious features, patients also exhibit different non-immunological features, including craniofacial, neurological, dental, vascular and musculoskeletal anomalies.

In 2004, Renner et al. [30] have described the case of a novel form of HIES, sharing some of the clinical features of the AD-HIES, but with an AR inheritance and a different profile of susceptibility to infections. In addition, these patients have a neurological involvement and a high predisposition to autoimmunity and proliferative disorders. The genetic defect was first identified in 2006, with the recognition of mutations in the *TYK2* gene [27]. In particular, the patient suffering from this variant showed alterations of the signaling pathway mediated by IFNα, IL-6, IL-10, IL-12 and IL23, resulting in an impairment of both innate and adaptive immunity. The TYK2 deficiency remains, however, a very rare form, whose clinical features are still controversial, as demonstrated from the description of the second case, with very different clinical presentation, characterized by disseminated BCG infection, recurrent zoster and neurobrucellosis in the absence of high levels of IgE [28].

On the other hand, many cases of AR-HIES have been ascribed to alterations in the *DOCK8* gene, which encodes a protein involved in the regulation of cytoskeleton [29]. Patients with *DOCK8* deficiency show a more severe phenotype than AD-HIES patients, characterized by severe viral infections primarily involving the skin (HPV, VZV, MCV), recurrent bacterial infections, severe atopy and high risk of early onset malignancies to the extent of 10–36% of patients. Notably, *DOCK8* deficiency may be associated with IgE levels within the normal range or only moderately elevated as compared with AD-HIES form. In addition, AR-HIES patients do not display somatic features, such as dental abnormalities, craniofacial or skeletal abnormalities, compared with the STAT3-dependent AD-HIES [30]. Eventually, neurological manifestations, such as facial paralysis, hemiplegia, cerebral aneurisms, and CNS vasculitis have been observed [30]. Within the malignancies, HPV-associated carcinomas, EBV-associated Burkitt lymphoma and diffuse large B cell lymphoma clearly predominate, not infrequently with an onset during the childhood [31].

A recent study described the case of a patient with Olmsted Syndrome due to transient receptor potential cation channel, subfamily V, member 3 (*TRPV3*) gene mutations, characterized by hyperkeratotic cutaneous lesions and palmoplantar keratoderma associated with a peculiar immunological and infectious phenotype characterized by high IgE levels, recurrent hypereosinophilia, increased IgA levels, reduced IgG3 subclasses and frequent skin infections sustained by bacteria and fungi, particularly by Candida albicans. The clinical phenotype is highly suggestive of a primary role of *TRPV3* gene, which is expressed in keratinocytes and langerhans cells of the skin in the immune response [32].

As a matter of fact, elevated serum IgE levels, although at a lower extent than HIES, are often found in many other PIDs, including Omenn Syndrome, due to hypomorphic mutations of *RAG1*, *RAG2*, *ARTEMIS*, *ADA* and *RMRP* genes; Wiskott–Aldrich Syndrome due to mutations in the *WAS* gene; atypical DiGeorge Syndrome with deletion of chromosome 22q11.2; IPEX Syndrome (immuno-disregulation,

polyendocrinopathy, enteropathy, X-linked) caused by mutation of the *FOXP3* gene and finally, Comel–Netherton Syndrome due to defect of *SPINK5* (Table 2) [33]. Each of these disorders shows a peculiar clinical phenotype that strongly distinguishes them from the classical forms of HIES. Such a strong association between the number of PIDs, whichever is the form, and the elevated IgE levels, would argue in favor of a still unappreciated biologic role for IgE in these patients and, in general, in the immune system physiology.

NUDE/SCID PHENOTYPE

Ectodermal Disorders and *FOXN1* Transcription Factor

As previously mentioned, ectodermal dysplasias include disorders sharing abnormalities of the skin, its appendages, such as hair, nails, teeth, sweat glands and sebaceous glands, and of other organs, which develop from ectoderm, such as the nervous system, the lens of the eye, and the mammary glands. These disorders may appear separately or together with other clinical manifestations involving mesoderm and endoderm [7]. Due to the huge number of different ectodermal dysplasias, there is a remarkable overlapping of clinical phenotypes.

A few overlapping signs with ectodermal dysplasia, associated with immunological abnormalities, are found in immunodeficiencies, such as those caused by the alterations of the *NEMO* gene [34], and of the RNA component of mitochondrial RNA processing endoribonuclease gene (*RMRP*), responsible for cartilage hair hypoplasia syndrome [35], or the *FOXN1* gene, the latter being responsible for the human or murine athymia, associated with the skin and hair defects, and, putatively, neural tube abnormal development [36, 37]. *FOXN1* gene is a "winged helix" transcription factor belonging to the forkhead-box gene family, which comprises genes implicated in a variety of cellular processes, such as development, metabolism, cancer and aging [38]. These transcription factors are developmentally regulated and direct tissue-specific transcription and cell fate decisions. In the pre-natal life, *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, while in the post-natal life *FOXN1* is expressed only in the epithelial cells of the skin and thymus. In the epidermis, *FOXN1* is expressed within differentiated epidermal and follicular cells. Differently, in the thymus, *FOXN1* acts early in the organ development, promoting TEC progenitor proliferation and directing specification of thymic epithelial precursor cells to cortical and medullary lineages [39–42]. The tissue specificity expression of *FOXN1* is probably due to the presence in its sequence of two exons, exons 1a and 1b, that undergo to alternative splicing to either of the two splice acceptor sites of the exon 2. The alternative usage of exon 1a or 1b is due to the presence of distinct promoters: promoter 1a, which is active in both the thymus and the skin, and promoter 1b, which is active only in the skin [43].

The Nude/SCID Syndrome and Its Associated Skin Abnormalities

The human Nude/SCID syndrome is characterized by the absence of a functional thymus, which results in a severe T-cell immunodeficiency [36]. This phenotype is the first example of SCID due to mutations of gene not expressed in hematopoietic cells [44].

Studies performed on human Nude/SCID fetus have added novel information on T-cell development in humans and, in particular, on the crucial role of *FOXN1* in early prenatal stages of T-cell ontogeny in humans. *FOXN1* gene mutations prevent the development of the T-cell compartment, affecting the $CD4^+$ cells more than the $CD8^+$ ones, as early as at 16 weeks of gestation [45]. Of note, in the absence of *FOXN1*, the thymic functionality is almost absent, as demonstrated by the absence of $CD4^+CD45RA^+$ naive cells [45]. However, very few $CD3^+CD8^+CD45RA^+$ naive cells

TABLE 2. Primary immunodeficiencies with elevated IgE levels.

Immuno deficiency	Inheritance	Gene	Immunological phenotype	Clinical features	OMIM
Omenn Syndrome	AR	Hypomorphic mutations of *RAG1/2*, *ARTEMIS*, *ADA* and *RMRP*, *IL7/Ra*, *DNA ligase IV*, γc, other unknown genes	Elevated IgE, reduced serum Ig levels, normal number of T lymphocytes with low heterogeneity, normal or reduced B lymphocytes	Erytroderma, eosinophilia, lymphoadenopathy, hepatosplenomegaly	#603554
Wiskott–Aldrich Syndrome	XL	*WAS*	Increased IgA and IgE, altered lymphocytic proliferative response, no or/low antibody response to polysaccaride antigens	Microtrombocytopenia, eczema, autoimmune disorders; viral and bacterial infections	#301000
Wiskott–Aldrich type 2	AR	*WIPF1*	Reduced B and T CD8 lymphocytes, low NK activity	Eczema, trombocytopenia, recurrent infections	#614493
Comel–Netherton Syndrome	AR	*SPINK5*	High IgE, reduced IgA levels, reduced switched and no-switched B lymphocytes	Ichtyosis, bamboo hair, atopy, increased susceptibility to viral and bacterial infections, growth retardation	#256500
IPEX	XL	*FOXP3*	Altered number and/or function of regulatory CD4+ CD25+ FOXP3+ T-cells, normal or elevated IgA and IgE levels	Autoimmune enteropathy, early-onset diabetes mellitus, eczema, autoimmune disorders	#304790
Olmsted Syndrome	AR	*TRPV3*	High IgE and IgA levels, reduced IgG3, hypereosinophilia	Palmo-plantar keratoderma, alopecia, onichodistrophy, recurrent fungal and bacterial cutaneous infections, squamous cell carcinoma	#614594

can be detected in the peripheral blood. Most of the T-cells bear TCR$\gamma\delta$ instead of TCR$\alpha\beta$ [45] and, although altered, the TCR gene rearrangement occurs in the absence of the thymus, suggesting an extrathymic site of differentiation for TCR chains, which is *FOXN1*-independent. Indeed, recent evidence suggests that, during embryogenesis, in the absence of *FOXN1*, a partial T-cell development can occur at extrathymic sites [46].

The Nude/SCID syndrome is more severe than the DiGeorge Syndrome, an immunodeficiency due to a complete or partial absence of the thymus, not associated with hairlessness or gross abnormalities in skin annexa. Peculiar features of the Nude/SCID syndrome are ectodermal abnormalities, such as alopecia and nail dystrophy [47]. The first identified mutation of *FOXN1* gene responsible in homozigosity for the disease is the C-to-T shift at 792 nucleotide position in the exon 4 (formerly exon 5) of the cDNA sequence. This mutation results in a non-sense mutation (R255X) and the complete absence of protein [48]. The second one, described in a French patient, is the C987T transition in exon 6, resulting in a mis-sense mutation (R320W) of the DNA binding domain [49]. A third novel mutation in *FOXN1*, resulting in TlowB+NK+SCID with alopecia [50] has been described recently.

The first two patients, identified by Pignata et al., in 1996, were two sisters with T-cell immunodeficiency and alopecia of the scalp, eyebrows, eyelashes and nail dystrophy [36]. Alopecia and nail dystrophy are also the characteristics of dyskeratosis congenita (DC) [51, 52], whose diagnostic criteria include a reticular pattern of hyper- and hypopigmentation of the skin, nail dystrophy, and mucosal leucoplakia [53]. However, in the two patients described, two diagnostic criteria of DC (abnormal pigmentation of the skin and mucosal leucoplakia) were lacking. Moreover, also the immunologic abnormalities were different from those associated with DC [54, 55].

The causal relationship between alopecia, nail dystrophy, and immunodeficiency does exist in Nude/SCID patients, and is also found in athymic mice that completely lack body hair (Table 3). Mice homozygous for the *FOXN1* mutation have retarded growth, decreased fertility, die of infections and are hairless (from which the term "nude" derived). The hairless feature is due to an abnormal epidermal developmental process, since in the skin of the nude mouse there are a normal number of hair follicles but not capable to enter the skin surface [56, 57]. In addition, the epidermis of the nude mouse fails to differentiate the spinous, granular and basal layers and shows a reduced number of tonofilaments. As in humans, also in mice the thymus is absent at birth [58], thus leading to a profound T-cell deficiency, which also affects humoral immunity. Of note, in a few strains of nude mice, alterations of digits and nails have been reported.

In 1999, a screening search for this *FOXN1* mutation in order to provide genetic counseling and prenatal diagnosis support to the community where the first patients were identified, led to the identification of healthy subjects carrying the heterozygous *FOXN1* mutation. These subjects were further examined for ectodermal alterations and showed nail abnormalities, such as the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate and the canaliform dystrophy associated to a transverse groove of the nail plate (Beau line), and the leukonychia (half-moon), characterized by a typical arciform pattern involving the proximal part of the nail plate [47]. This is not surprising, since *FOXN1* is selectively expressed in the nail matrix where the nail plate originates, and where it is involved in the maturation process of nails. In keeping with the expression of *FOXN1* in the murine epithelial cells of the developing choroids plexus, a structure filling the lateral, third, and fourth ventricles, additional studies revealed, in human Nude/SCID aborted fetus, the presence of severe neural tube defects, including anencephaly and spina bifida. However, since the anomalies of brain structures have been reported only inconstantly, this

TABLE 3. Main similarities shared between human Nude/SCID and murine "nude" phenotype.

	Human Nude/SCID	Nude mouse
Clinical features		
Thymus absence	+	+
Retarded growth	+	+
Omen-like syndrome	+	-
Severe infections	+ (interstitial pneumopathy)	+
Neural tube defects	+ (anencephaly and spina bifida)	-
Severe infertility	Unknown	+
Small ovaries with low eggs count (female)	Unknown	+
Motile sperm absence (male)	Unknown	+
Altered serum levels of estradiol, progesterone and thyroxine	Unknown	+
Immunological features		
Presence of normal T-cell precursors	+	+
Lymphopenia	+ (T-cells)	+ (T-cells)
Absence of specific thymus-derived cells	+	+
Absence of proliferative response to mitogens	+	+
Very few lymphocytes in the thymus-dependent areas of the spleen and the lymph node	+	+
Presence of antibody forming cell precursors	+	+
Low levels of serum immunoglobulins	+	+
Very low/absent production of specific antibodies	+	+
Skin and skin annexa features		
Hairlessness	+ (alopecia of the scalp, eyebrows and eyelashes)	+
Alterations of digits and nails	+ (leukonychia, koilonychias, canaliform dystrophy)	+
Unbalance between proliferation and differentiation of keratinocytes in the hair follicle	+	+
Coiling of incomplete hair shafts in the dermis	+	+

would suggest that *FOXN1* plays a role of a cofactor only in brain development during embryogenesis [37].

SKIN ELEMENTS TO SUPPORT T-CELL ONTOGENY

Given the association between skin developmental alterations and immunodeficiencies, a possible explanation is that a remarkable number of similarities are shared between the epidermal and the thymic epithelium. Similarities between the human thymic epithelial cells (TECs), a key cell component of the thymic stroma, and human

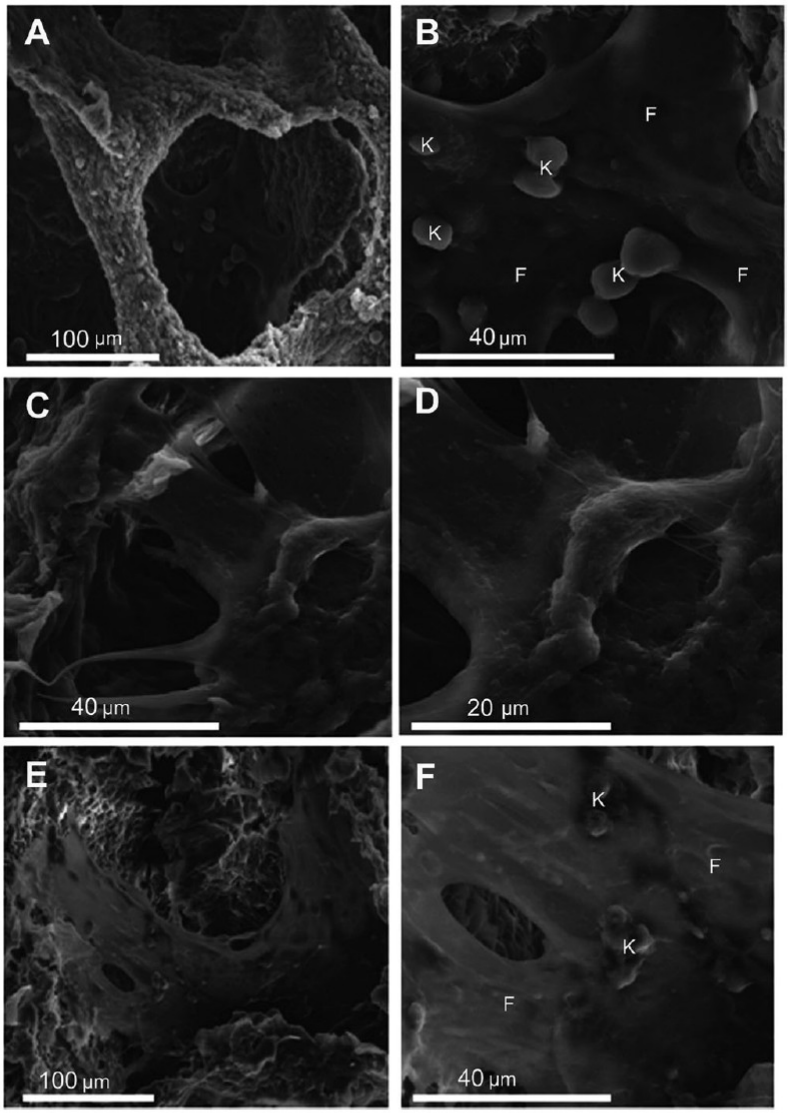

FIGURE 1. Representative scanning electron micrographs of keratinocytes and fibroblasts co-cultured on the PCL scaffold. Expanded fibroblasts and keratinocytes were seeded together onto artificial 3D PCL scaffolds, and after cell infiltration, the structure was studied by scanning electron microscopy (SEM). (A) Each cell type established physical contacts with the PCL scaffold and organized on the surface of its inner pores. (B) In particular, a strong interaction of keratinocytes with fibroblasts occurred. (C and D) Fibroblasts formed focal adhesions with the matrix, thanks to their thin filopodi (D). After 3 weeks of culture, interaction of each cell type with material (E) and with each other (F) was clearly visible.

keratinocytes were identified through comparison of gene and protein expression and *in vitro* analysis [59, 60]. The development of both epidermal and thymic epithelium requires the expression of p63, the p53 family transcription factor [61–63], which was earlier expressed in the development of both epithelial lineages [63–65]. Epidermal development is abrogated in mouse models with the loss of p63 function, resulting in few keratinocytes and lack of stratification, which causes rapid dehydration and early postnatal lethality of these mice [66, 67]. A similar epithelial phenotype occurs in p63−/− mice's thymus, which showed defects in the proliferative rate of TECs that leads to thymic atrophy.

Along with *FOXN1*, another transcription factor is shared between epidermal progenitor cells in the epidermis and the thymus, the T box gene *Tbx1*. The absence of *Tbx1* results in the loss of hair follicle stem cell renewal in the epidermis [68], and in the loss of thymic epithelial development [69–71]. Furthermore, thymic stroma and skin elements also share the Notch pathway, which plays an important role in the regulation of epidermal differentiation [72]. Within the thymus, it is necessary for T-cell linage commitment and the early stage of thymocyte maturation [73].

A major regulator of both hair follicle placode formation and thymic epithelial development is the fibroblast growth factor (FGF) signaling pathway [74, 75]. Several studies revealed the importance of FGFs mesenchymal expression to promote epithelial proliferation and invagination to generate mature thymic rudiments and epidermal hair placodes [76, 77].

Additional similarities between the two organs concern the cellular organization, in that medullary TECs are able to form Hassall's corpuscles, following a developmental program, analogous to skin epidermal basal cells, which form cornified cells [60]. Furthermore, proliferating TECs, derived from rats transplanted into the skin, formed epidermis and skin appendages such as the sebaceous gland and hair follicle [61], highlighting the responsiveness of TECs to the skin tissue environment.

Thus, although the functions of the skin and thymic epithelial components are quite distinct, both tissues have primary roles in establishing immunity [78]. TECs create an environment that promotes the expansion, maturation and specification of immature T cells. Epidermal keratinocytes are also essential for driving the activation of the innate and adaptive immune system through the production of cytokines, which direct the fate of discrete lymphocyte populations, known as the "epimmunome" [79].

Not by chance, recently it has been demonstrated that a co-culture of human skin-derived keratinocytes and fibroblasts, in the absence of thymic components, can support the survival of human hematopoietic stem cells and their differentiation into T-lineage committed cells [80], suggesting that skin keratinocytes can promote T-cell development, such as TECs, although at a low efficiency (Figure 1).

In addition, it has been shown that murine skin fibroblasts, enforced by *FOXN1* expression, are able to reprogram into induced TECs (iTECS), an *in vitro* generated cell type that exhibits phenotypic and functional properties of *in vivo* TECs. iTECS are able to promote full T-cell development *in vitro*, providing the basis for thymus transplantation therapies aimed at boosting adaptative immune system function in immunocompromised patients [81].

CONCLUSIONS

Primary immunodeficiencies are severe early onset immunological disorders often fatal in the first years of life. Many forms show cutaneous features in association with immunological defects. In fact, the presence of skin and skin annexa abnormalities may be considered a warning sign in patients with a suspicion of a primary immunodeficiency. In this review, we focused on the most common forms of PIDs associated with

ectodermal disorders, highlighting the alarm signs that should lead the clinician to consider a deeper immunological assessment, investigating both molecular and functional aspects. Moreover, this approach would be very helpful in the early detection and treatment of such complex disorders.

Declaration of Interest:

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

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3.2 Molecular evidence for a thymus-independent partial T cell development in a FOXN1-/- athymic human fetus

The Nude/SCID Syndrome (MIM 601705; Pignata Guarino Syndrome), is due to genetic alterations of the FOXN1 transcription factor, that results, both in mice and humans, in congenital athymia and hairlessness. This autosomal recessive disorder, leads to a severe T-cell immunodeficiency, congenital alopecia of scalp, eyebrows and eyelashes. This phenotype, referred as Nude/SCID, was described for the first time in humans in 1996 in two sisters originating from a small community in the south Italy (106). This phenotype is widely accepted as the human equivalent of the similar murine phenotype, reported for the first time by Flanagan in 1966 (107). Evidences suggest a T-cell differentiation at extrathymic sites, as intestine and liver and tonsil, where probably T cell populations may arise from preexisting precursor cells (108-112).

Recently, our group demonstrated that gut represents an alternative site of T-cell ontogeny in a FOXN1-independent and thymus-independent fashion. Taking advantage of the opportunity to study tissue samples from an athymic human Nude/SCID fetus, we demonstrate that, a few rare T lymphocytes are detectable in the cord blood, even though a complete blockage of the CD4+ population occurred (113). In the Nude/SCID fetus, most of the rare CD3+ cells were CD4-CD8-. Moreover, similarly to a control fetus, a CD3-CD7+CD2+ population was found, suggesting that the differentiation of this population is FOXN1-independent. In human fetal gut, part of the CD3-CD7+ population develops into CD3+ T cells presumably in a thymus-independent manner (114).

It is known that T-cell and thymus-independent NK-cell precursors express the CD7 molecule in bone marrow and liver from the 5th week of gestation (115),

then, CD7+ cells migrate to the thymus to become fully mature T cells (116). In the absence of the thymus, it is plausible that this population, which also express CD2 molecule, could originate from extrathymic sites. CD7+CD2+CD3-lymphocytes have already been documented in the epithelium of the small intestine (116). However, the expression of CD7 but not of the CD2 marker suggests a T-cell lineage commitment. In the Nude/SCID and the control fetuses, a CD3-CD7+CD2- population was identified, thus supporting the hypothesis that this precursor differentiates toward the T-cell rather than NK-cell lineage.

There is also evidence indicating that CD3+ mature cell population originates from CD3-CD7+ population that express the CD8αα homodimer (114). The expression of the CD8αα homodimer has only been documented in mice on extrathymic derived intraepithelial lymphocytes (IELs) (117), but it is not clear if this population is produced in the intestine or rather they require a functional thymus to be generated. In our Nude/SCID fetus we found a negligible number of cells expressing the CD8αα homodimer, thus indicating the requirement of an intrathymic step of differentiation for these cells.

In our study, we supported the evidence of the local production of T lymphocytes in the intestine in a thymus independent fashion by demonstrating the presence of CD3+ and few CD8+ cells in the fetal intestin of the Nude/SCID fetus, and of CD3ε transcript, indicating that the process if FOXN1-independent.

The hallmark of the T-cell ontogeny is the TCR gene rearrangement process for V(D)J, characterized by distinctive patterns of gene expression, such as RAG1 and 2 in lymphocytes of both T- and B-cell lineages, which are responsible for the DNA cleavage (118).

The T-cell commitment is established by the presence of pTα transcript (119)-. This molecule associates with the mature TCR α-chain allowing rearrangement of the α-chain gene (120). The expression of pTα and RAG genes is upregulated during the DN3 stage of the T-cell development also in IELs RAG1, indicating that TCR gene rearrangement occurs locally in the intestine (121). In our study we found through Real Time PCR, that the relative expression of RAG1 and RAG2 was approximately 50% of the control while pTα expression was 10% of the control, demonstrating a thymus-independent T-lymphopoiesis in the intestine of the Nude/SCID fetus. Moreover, we previously documented an impairement of the 2 Vβ families in the Nude/SCID cord blood. In this study, we found that the spectratype of intestinal T cells paralleled the cord blood spectratype in that all the families expressed in the Cord Blood Mononuclear Cells (CBMC) were also expressed in the intestine. This finding indicates that, in the absence of the thymus, the development of intestinal lymphocytes only expressing a limited repertoire occurs.

The expression of CD103 integrin helps the migration of the previousely described lymphocytes into the intestine by interacting with the E-cadherin ligand expressed on epithelial cells (122). In this study, we didn't detect CD103 marker in the intestine suggesting that the lymphocytes present in the intestine were not elsewhere originated cells migrated into the intestine.

Collectively, the results of this study represent the first formal demonstration that an extrathymic lymphopoiesis in the absence of the thymus and in a FOXN1-independent manner occurs in humans, but this is not able to provide a productive immune response in the peripheral blood.

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

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

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Abstract

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

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Introduction

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

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

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

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

Results and Discussion

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

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

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

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

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

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

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

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

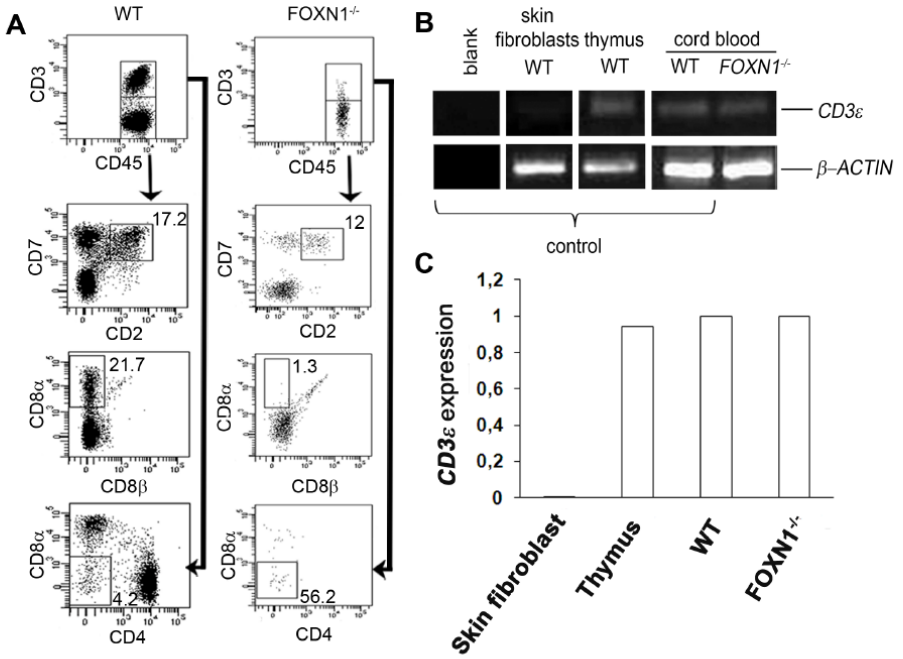

Figure 1. Detection of extrathymically derived T lymphocytes in the cord blood of $FOXN1^{-/-}$ human fetus. (A) Flow cytometry analysis of CBMCs from WT (left dot plots) or $FOXN1^{-/-}$ (right dot plots) fetuses (16 weeks of gestation). CD7 and CD2 together with the CD8α and CD8β expression patterns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

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

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

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

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

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

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

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

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

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

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

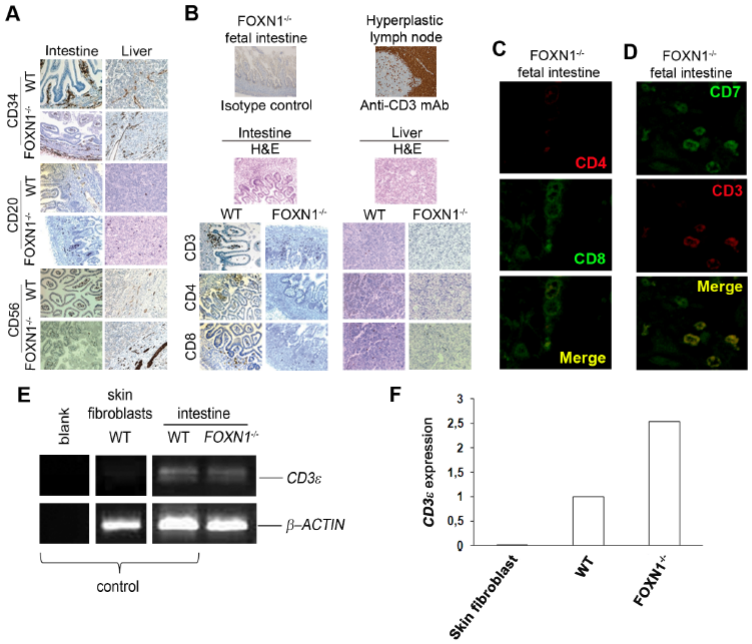

Figure 2. Identification of lymphocytes at extrathymic sites of differentiation in a Nude/SCID human fetus. (A, B) Immunohistochemical detection of lymphocytes at extrathymic sites of differentiation in FOXN1$^{-/-}$ human fetus (16 weeks of gestation). (A) Stem cells, B cells and NK cells were detected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

Materials and Methods

Fetus samples

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

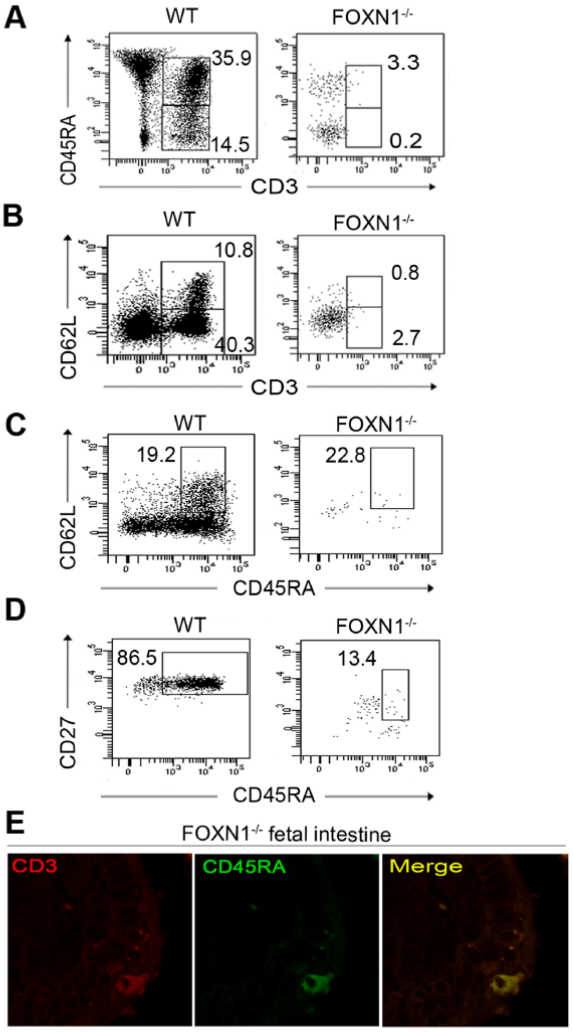

Figure 3. Lymphocytes with naive phenotype in cord blood and intestine. Flow cytometry of CBMCs from normal and FOXN1$^{-/-}$ fetuses matched for gestational age (16 weeks of gestation). Dot plots show the expression pattern of the naïve cell markers. (A) Frequencies of CBMCs expressing both CD45RA and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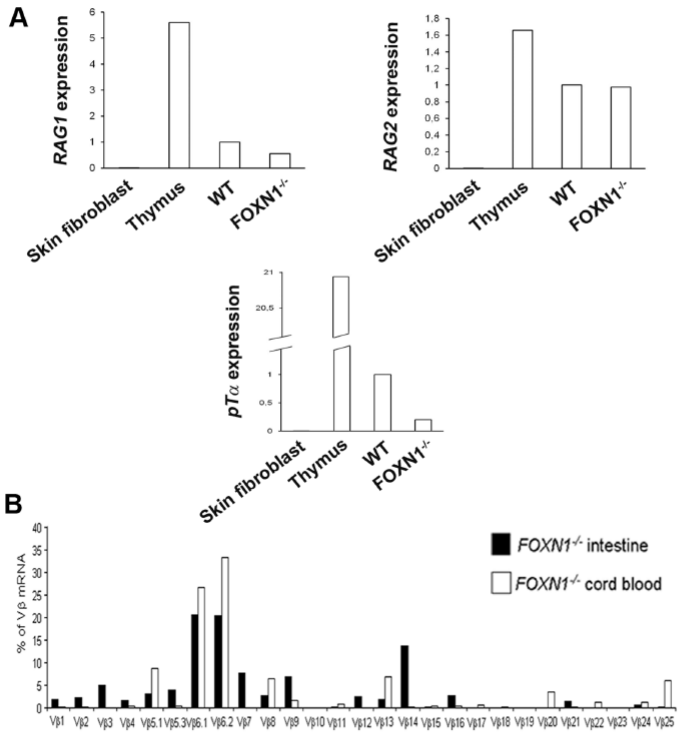

Figure 4. Local production of T cells in the FOXN1$^{-/-}$ human intestine. (A) Quantitative real-time PCR showing the expression of mRNAs encoding RAG1, RAG2 and pTα (relative to β-actin) in skin fibroblasts (negative control), thymus (positive control) and intestinal tissue of control and FOXN1$^{-/-}$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

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

Histology

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

Immunohistochemistry

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

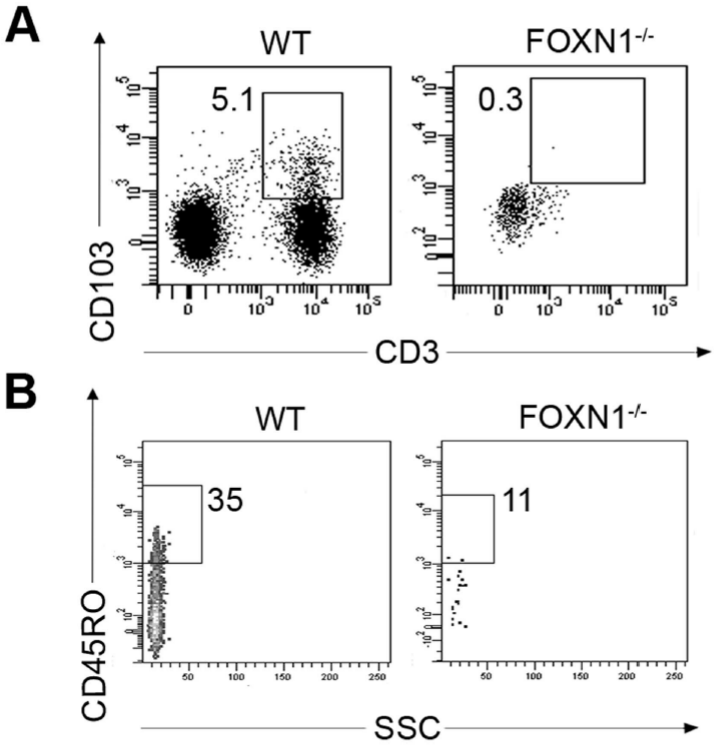

Figure 5. Expression on T lymphocytes from FOXN1$^{-/-}$ and control fetuses of the integrin CD103 and the CD45RO activation marker. (A, B) Flow cytometry of CBMCs from normal and FOXN1$^{-/-}$ fetuses matched for gestational age (16 weeks of gestations). Dot plots show the frequency of CD103$^+$CD3$^+$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

Confocal microscopy

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

RNA and RT-PCR

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

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

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

Quantitative real-time PCR

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

Statistical analysis

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

Acknowledgments

This study is dedicated to Salvatore Auricchio and Armido Rubino for their full commitment to Research in Pediatrics.

Author Contributions

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

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3.3 APECED: a paradigm of complex interactions between genetic background and susceptibility factors

Autoimmune Polyendocrinopathy Candidiasis Ectodermal Distrophy (APECED) is a rare autosomal recessive disease (OMIM 240300) which affects many tissues especially endocrine glands (123). APECED is caused by mutations in the AutoImmune REgulator gene (AIRE), which maps to 21q22.3 and encodes a 55-kDa protein that acts as a transcription regulator (123). AIRE is predominantly expressed in thymic epithelial cells. It is also expressed in monocyte-derived cells of the thymus, and in a subset of cells in lymph nodes, spleen and fetal liver. A great contribution to the current knowledge of the pathogenesis and the clinical hallmarks of the syndrome came out also from the large number of studies performed in AIRE knockout mice. These mice display signs of immunological dysregulation as T-cell hyperreactivity, perturbation of the TCR repertoire (124).

The diagnosis is primarily based on the presence of two out of the three most common clinical features: hypoparathyroidism, Addison's disease, and chronic mucocutaneous candidiasis (123). Further clinical or latent autoimmune endocrine signs or diseases may be associated. They include hypergonadotropic hypogonadism, Type 1 diabetes mellitus, and autoimmune thyroid disease. Non-endocrine autoimmune disorders include vitiligo, alopecia, urticaria-like erythema, chronic atrophic gastritis with or without pernicious anemia, celiac disease, malabsorption, autoimmune hepatitis, rheumatic diseases. Finally, other clinical features such as cholelithiasis, ectodermal dystrophy, acquired asplenia, cancer of the mucosae, calcifications of basal ganglia and tympanic membranes may also occur (123). The presence of chronic Candida infection suggests a T-cell defect toward intracellular pathogens as documented by the defective T cell response in patients with APECED.

However, the expression of the clinical phenotype is often partial in infancy. In fact, most of the patients with the classic triad of symptoms belong to the second or third decade of life. In infancy, chronic candidiasis is generally the presenting sign of the syndrome, usually appearing before the age of 5 years. Unfortunately, children suffering from isolated candidiasis are not investigated for AIRE mutations. This leads to underestimate the prevalence of the syndrome in infancy. It is often followed by chronic hypoparathyroidism, before the age of 10 years, and later by adrenal insufficiency. Further associated diseases may appear from childhood up until the fifth decade of life (125).

Although APECED is the first well documented example of autoimmune disorders inherited as a monogenic disease, there are several genetic or environmental factors which influence the onset of the disease and its clinical expression. In fact, APECED patients show a variable range of pathological manifestations, with each patient presenting a different constellation of affected organs and autoantibodies specicfiities. Furthermore, the severity of each individual manifestation may profoundly differ between patients carrying the same mutation.

Correlation studies so far failed to reveal a correlation between phenotype and genotype and, even among siblings with the same genotype, clinical phenotype can reveal wide heterogeneity. These data suggest that factors other than the diversity of mutation in the AIRE gene affect the phenotype. Associations to specific HLA aplotypes have been found for trait components like alopecia, Addison's disease and type 1 Diabetes in APS 1. The associated haplotypes are the same that associate with the common, non-APS-1 related forms of these diseases. Only a weak association have been observed between the HLA type and autoantibody specificities in APS-1

patients, suggesting that in APS-1 the HLA alleles do not have a strong influence on autoantibody formation (126).

Along with the central tolerance network, several mechanisms are capable to contribute in controlling and regulating in the periphery the immune system. These factors are involved in the maintenance of homeostasis of peripheral tolerance of residual autoreactive clones which escape negative selection within the thymus and play a significant role to prevent or minimize reactivity to self-antigens. Peripheral tolerance is attributable to the induction of functional anergy, deletion by apoptosis and the suppressive action of regulatory T lymphocytes (Treg). Anergy is a mechanism that results in functional inactivation of self-reactive T cells. Clonal T cell anergy can be induced upon engagement of the TCR in the absence of co-stimulatory signals. Apoptosis takes place through a series of regulated biochemical events that follow Fas/FasL interaction, thus resulting in cell death. Treg cells arise during the normal process of maturation within the thymus (127) and preferentially express high levels of CD25, the transcription factor forkhead box P3 (FoxP3) and a considerable number of additional activation surface markers, as transferrin receptor and HLA class II antigens. These cells exhibit a vast spectrum of autoimmunity-preventive activity. Treg cells are naturally anergic and, upon TCR activation, potently suppress the proliferation of CD4+CD25- T cells through an antigen-nonspecific mechanism (128). Moreover, the intimate molecular mechanism by which Treg cells mediate suppression still remains unclear. An additional mechanism involved in controlling reactivity to self engages in the periphery Natural Killer cells (NK) activity. There is strong evidence that clearly show the association between low levels of NK cells and NK-cell activity and the development of autoimmunity, attributable to failure in deletion of autoreactive clones by cytolisis.

An alteration of the fine tuning of one of these processes leads to autoimmunity.

A focus on thecomplex pathogenesis of APECED and on the potential interfering factors involved in the clinical expression of the disease, has been published as Review on *Frontiers in Immunology,* for the manuscript see below. This review is part of an Issue we hosted, entitled "*Thymic stromal alterations and genetic disorders of immune system"*, for the Editorial see below.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
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APECED: a paradigm of complex interactions between genetic background and susceptibility factors

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Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal recessive disease, caused by mutations of a single gene named Autoimmune regulator gene (AIRE) which results in a failure of T-cell tolerance. Central tolerance takes place within the thymus and represents the mechanism by which potentially auto-reactive T-cells are eliminated through the negative selection process. The expression of tissue-specific antigens (TSAs) by medullary thymic epithelial cells (mTECs) in the thymus is a key process in the central tolerance and is driven by the protein encoded by AIRE gene, the transcription factor autoimmune regulator (AIRE). A failure in this process caused by AIRE mutations is thought to be responsible of the systemic autoimmune reactions of APECED. APECED is characterized by several autoimmune endocrine and non-endocrine manifestations and the phenotype is often complex. Although APECED is the paradigm of a monogenic autoimmune disorder, it is characterized by a wide variability of the clinical expression even between siblings with the same genotype, thus implying that additional mechanisms, other than the failure of Aire function, are involved in the pathogenesis of the disease. Unraveling open issues of the molecular basis of APECED, will help improve diagnosis, management, and therapeutical strategies of this complex disease.

Keywords: autoimmune polyglandular syndrome type 1, APECED, autoimmune regulator gene, phenotypic variability, tolerance

INTRODUCTION

Autoimmune Polyglandular Syndrome Type 1 (APS-1), also called Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), is a rare autosomal recessive disease caused by mutations of the autoimmune regulator gene (AIRE). Immunologically, APECED is characterized by destruction of the target organs by a cellular- and/or antibody-mediated attack (1). In the past decade, much interest has been focused on the pathogenesis of this syndrome. Indeed, APECED represents a paradigm of genetically determined systemic autoimmunity. However, the great variability that characterizes APECED, irrespectively of the AIRE genotype, implies that several factors are involved in the disease phenotypic expression.

In this review, we will focus on the complex pathogenesis of APECED and on the potential interfering factors involved in the clinical expression of the disease.

THE BASIS OF THE IMMUNOLOGICAL TOLERANCE

Tolerance represents a state of immunologic non-responsiveness in the presence of a particular antigen. In this context, T-cell tolerance is crucial for the creation of a proper T-cell repertoire, able to respond to a huge number of foreign antigens, but preventing autoimmune reactions. Imposition and regulation of self-tolerance within the T-cell repertoire is exerted at two levels: (1) central tolerance (development and selection of T-cells in the thymus) and (2) peripheral tolerance (deletion, anergy of mature T-cells in lymphoid and non-lymphoid organs) (2).

T-cell central tolerance, established within the thymus, mostly relies on two main mechanisms: negative selection, also referred to as clonal deletion of maturing thymocytes and positive selection of maturing T-cells able to bind to a surface major histocompatibility complex (MHC) molecule with mild threshold of reactivity (**Figure 1**). The thymus provides the necessary environment for thymopoiesis and establishment and maintenance of self-tolerance (3–5). Thymus contains thymic epithelial cells (TECs) that form a complex three-dimensional network organized in cortical and medullary compartments (6). On entering the thymus, immature thymocytes promote the differentiation of precursor thymic epithelial cells (pTECs) into cortical TECs (cTECs) and medullary TECs (mTECs), playing an important role in the formation of the thymic microenvironment (7–9). During postnatal life, hematopoietic progenitors enter the thymus from the bloodstream (10) and cells committed to the T lineage undergo division, mostly within the double-negative (DN) stage of the T-cell development. The first checkpoint is the rearrangement of T-cell receptor (TCR) β and α locus. Expression of αβ TCR heterodimers on the cell surface allows DN thymocytes to progress to the double-positive (DP) CD4+CD8+ stage. At DP stage, the TCR affinity for self-peptide-MHC on mTECs within the thymus determines thymocyte's fate. mTECs express a wide array of tissue-specific antigens (TSAs) in the context of MHC class II molecules; these TSAs include self-proteins derived from different organs in the body. DP thymocytes expressing TCRs that do not bind self-peptide-MHC complexes are programed to undergo "death by

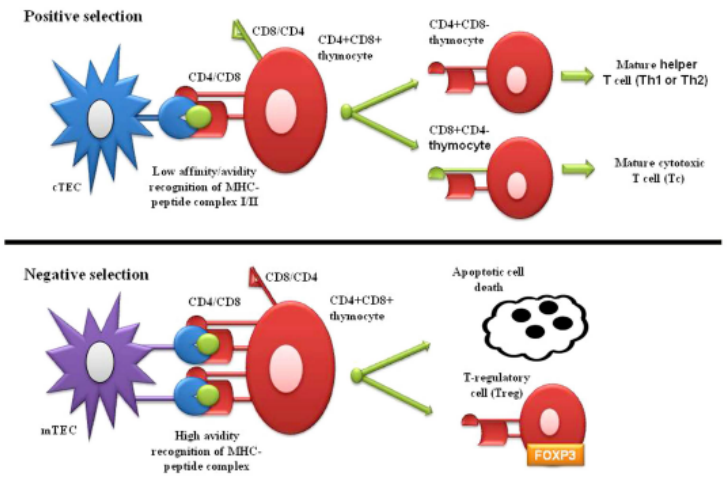

FIGURE 1 | Positive and negative selection of immature thymocytes within thymus.

neglect" or apoptosis. Only about 5% of DP has a low affinity for self-peptide-MHC complexes and differentiate to CD4+CD8− or CD4−CD8+ single positive (SP) lineage (positive selection) (11–13). DP thymocytes with high-affinity TCR for MHC complexes represent a potential reservoir of auto-reactive lymphocytes and "clonal deletion" (negative selection) is the main mechanism in the thymus to preserve self-tolerance (14, 15). Compelling evidence indicates that an altered promiscuous thymic expression of TSAs leads to autoimmunity. In the autoimmune attack, T helper cells (Th) escaped to self-tolerance, produce pro-inflammatory cytokines able to begin inflammation and activate auto-reactive B-cells, resulting in autoantibodies production, which lead to tissue inflammation and damage (1). Some of the thymocytes that recognize self-peptide-MHC complexes with high-affinity express Foxp3 and through "clonal diversion" mature as regulatory T-cells (Tregs), which are able to suppress auto-reactive T-cells in the periphery (16–18). The central tolerance is not able alone to completely remove mature T-cells with self-antigens specificity, therefore additional mechanisms in the periphery are also needed to maintain immunological tolerance.

The peripheral tolerance recognizes as possible mechanisms the induction of functional anergy, deletion of auto-reactive clones, and the suppressive action of T-regulatory cells (Tregs). Anergy is a state of long-term hyporesponsiveness with inactivation of self-reactive T-cells in the presence of a TCR signal but in the absence of a second costimulatory signal, necessary to T-cell activation. Deletion of self-reactive lymphocytes is achieved in both the thymus and the periphery by apoptosis through interaction of Fas/FasL. The function of Tregs (Foxp3-expressing CD4 T-cells) is to suppress immune responses through numerous mechanisms including the production of anti-inflammatory cytokines, direct cell–cell contact, and by modulating the activation state and function of antigen-presenting cell (APC) (19). An additional mechanism involved in controlling reactivity to self in the periphery is NK cell activity.

AIRE AND THE MAINTENANCE OF IMMUNOLOGICAL TOLERANCE

Autoimmune regulator gene encodes for a transcription factor (Aire) involved in the maintenance of tolerance. In humans, the AIRE gene maps to chromosome 21q22.3 (20, 21). It consists of 14 exons spanning 11.9 kb of genomic DNA (22) and encodes a 545 amino acid protein with a molecular weight of 58 kDa that works as a "non-classical" transcriptional factor in immune-related organs. The highest level of AIRE expression has been detected within the thymus (23) in mTECs, followed by thymic dendritic cells (DCs). In addition to the thymus, low level of Aire seems to be expressed in secondary lymphoid organs, such as lymph nodes, fetal liver, and spleen (24, 25). The Aire protein, mostly localized in the cell nucleus, is composed by specific domains including the amino-terminal HSR domain, the nuclear localization signal (NLS), the Sp100, AIRE1, nucP41/75, DEAF 1 (SAND) domain, two plant homeodomain (PHD) type zinc fingers, and four LXXLL motifs (26) (**Figure 2**). The HSR region has been shown to be responsible for the dimerization of the polypeptides belonging to the Sp100 protein family (27). The SAND domain is important for AIRE transactivation capacity and subcellular localization. The PHD zinc fingers are often found in proteins involved in the regulation of transcription (28). The LXXLL motifs are found on coactivators nuclear receptors and proline-rich regions (PRR) and are also

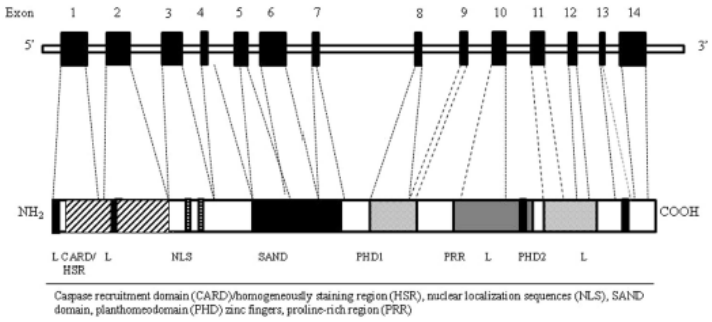

FIGURE 2 | AIRE gene (top) and corresponding protein (bottom) with functional domains. The Aire protein is composed by nuclear localization sequences (NLS), two planthomeodomain (PHD) zinc fingers, a caspase recruitment domain (CARD)/homogeneously staining region (HSR), a SAND domain, and a proline-rich region (PRR).

associated to transcription regulation (29). Although the precise molecular mechanism is still unclear, Aire seems to regulate the transcription process acting as a coactivator in a large transcriptional complex (30), and interacting with a large set of partners, divided into four main classes based on their function: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing factors (31). The first protein reported to bind to AIRE was CREB-binding protein (CBP) (32). Its interaction with AIRE may lead to promotion of gene transcription through histone acetylation and the recruitment of chromatin-transcription factors (33). Other AIRE partners have been identified, such as DNA protein kinase (DNA-PK), SP-RING domain protein inhibitor of activated STAT1 (PIAS1), positive transcription elongation factor b (P-TEFb) (34–36). Moreover, it has been proposed a possible epigenetic control of the AIRE target genes since AIRE's PHD1 finger domain appears to be able to bind histone three molecules with unmethylated lysine at position 4, generally associated with repressed genes (37). Overall, it is possible that Aire mediates the expression of TSA in mTECs through its co-transcriptional partners (38). The intriguing question is how the AIRE gene alone can influence the transcription of such a large number of TSA genes. Indeed, two models have been suggested to explain the action of Aire: transcription model and maturation model. In the transcription model, TSAs are considered to be the direct target genes of Aire's transcriptional activity and the lack of Aire protein within the cell would result in the defective TSA gene expression, while the maturation program of mTECs would be in principle unaffected. The maturation model suggests that Aire may affect the thymic microenvironment more globally than through simple control of TSA expression levels. Consequently, in keeping with the latest model the regulation of TSA gene expression might not be the major defect of Aire-deficient mTECs responsible for impaired negative selection (39).

Although the exact role of AIRE in controlling T-cell tolerance is still largely unclear, several mechanisms have been suggested. Functional alterations of AIRE may affect processing and/or presentation of self-antigens within the mTECs (40). The process of thymocyte maturation (41), the attraction of mature thymocytes to their final location for a proper negative selection (40, 42), the control of cross-presentation through alteration of the relationship between APCs and mTECs (43) may also represent potential mechanisms by which AIRE alterations may lead to functional abnormalities of the central tolerance. The alteration in the balance between negative selection and regulatory T-cell production (44) may also be implicated in the pathogenesis. In addition, Aire may also play a role in the proper differentiation of the thymic medullary epithelium, in the induction of apoptosis in end-stage terminally differentiated mTECs (39) as well as in mTECs' differentiation program. In particular, evidence suggests that lack of Aire in mTECs results in an arrest of the differentiation program, with the cells remaining at the premature stage just before terminal differentiation (45, 46).

THE CLINICAL COUNTERPART OF AIRE MUTATION: APECED

GENETIC BACKGROUND

Mutations in AIRE gene result in development of APECED, which represents the paradigm of a genetically determined failure of central tolerance leading to autoimmunity (46). APECED is a rare autoimmune syndrome, but it has been reported worldwide showing a relatively higher prevalence in genetically isolated populations such as Iranian Jews (1:9,000) (47), Finns (1:25,000) (48, 49), and Sardinians (1:14,400) (50). It is also quite frequent in Norway (1:90,000) (51) and in some regions of Italy (52–55). The most frequent model of inheritance is autosomal recessive, even though a dominant pattern has also been sporadically reported (56). So far, over 70 different mutations of AIRE have been documented (2). Due to the molecular organization and the complexity of intermolecular connection of Aire, it would be expected that different mutation in the molecule might imply different functional abnormalities, thus being associated with a variable phenotypic expression. Single nucleotide substitutions, small insertions, deletions, and mutations affecting splice

consensus sequences have been identified along the entire coding region, and include either nonsense or frameshift mutations that result in truncated polypeptides, or missense mutations that result in single amino acid-changing (27). Most of these AIRE mutations lead to a change in its subcellular location altering the distribution of the protein between the nucleus and cytoplasm (27). Mutations of the predicted surface area of the HSR domain cause the protein accumulation in the nucleus blocking its cytoplasmic localization probably enhancing nuclear import or inhibiting nuclear export (57). Mutations of the SAND domain disturb the distribution of Aire between the nucleus and cytoplasm suggesting a role for the SAND domain in nuclear transport mechanisms (57). Moreover, since the six helix CARD domain is involved in homodimerization, missense mutations in this region often affect Aire multimerization or localization to nuclear bodies (58) while most of the missense mutations in PHD domains alter the zinc-finger fold and decrease Aire's transcriptional activation capacity (38).

Some different mutations have been found to be peculiar to certain populations. R257X is the most common mutation among Finnish and other European patients (59–61). R257X is a nonsense mutation, which most probably results in a carboxy-terminally truncated, non-functional Aire protein leading to alterated subcellular localization and inhibition of the transactivation function and complex formation of Aire (27). The 1094–1106 del113 (or 967–979 del-13 bp) is the most common mutation in British (62), Irish (63), North American (64, 65), and Norwegian patients (51) leading to the truncation and loss of function of Aire. Y85C is the only missense mutation found among Iranian Jews (57). In Italy, typical mutations of AIRE have been detected in Sardinia (R139X on exon 3) (50, 54), where this nonsense mutation leads to a total absence of Aire and seems to be associated with a more severe phenotype. In Apulia, the missense mutation W78R on exon 2, and the nonsense mutation Q358X on exon 9 have been found. The mutation Q358X lies in the PRR resulting in a truncated protein which lacks the second PHD finger and thereby is most likely non-functional protein (53). In Sicily, the most frequent mutation is R203X on exon 5, and two novel mutations, S107C and Q108fs on exon 3, have been detected. The mutation S107C is a missense mutation, whilst Q108fs is a small deletion, both affecting the HSR domain of Aire protein and it is likely that the Aire protein loses its homodimerization properties (66, 67). In Venetian patients, the most frequent mutations are R257X on exon 6 and 979 del-13 bp on exon 8, that are analogous to those detected in Finnish and Anglo-saxon patients but different from Italian ones (55). No typical mutations have been identified neither in Calabria nor in Campania (52, 68) even though patients from Campania show a high frequency of mutations in the exon/intron 1 junction. Compared to other mutations, the R257X results in a total loss of function, whereas the less dramatic truncations of the AIRE protein and many missense mutations, especially the predicted surface mutations of the HSR domain and the mutations in the leucine zipper domain, seem to exert less severe effects on the function of the Aire protein (27). Therefore, despite considerable variations in the APECED genotype, correlations with specific phenotypic features are far from being well elucidated. Only in patients affected with Candida infection, a correlation has been proved. In fact, candidiasis was significantly less prevalent in patients homozygous for 967–979del-13bp than in patients carrying the R257X or R139X, suggesting that AIRE truncation upstream the SAND domain promotes the susceptibility to this infection (69).

DIAGNOSIS OF APECED

The onset of APECED usually occurs during childhood. The clinical diagnosis is based on the presence of two of the three classical components: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison's disease (AD). The presence of only one of these features is sufficient for the diagnosis, when a sibling is affected. Molecular analysis of AIRE may help to confirm the clinical diagnosis, in particular in those cases with an atypical presentation (70, 71). Neutralizing autoantibodies against IFN-ω and IFN-α may represent a precocious biomarker detectable in the majority of patients and, thus they have been recently included in the diagnostic criteria of APECED (72).

CLINICAL EXPRESSION, AUTOANTIBODIES PROFILE, AND SUSCEPTIBILITY FACTORS

APECED is characterized by a highly variable pattern of destructive autoimmune reaction, mainly mediated by specific autoantibodies toward different endocrine and non-endocrine organs. Virtually, all tissues and organs may represent the target of the autoimmune attacks, thus leading to a wide spectrum of clinical features. As already mentioned, the three main components of APECED are CMC, CH, and AD. CMC is, generally, the first component to develop, often followed by CH, before the age of 10 years and later by adrenal insufficiency (73, 74). In addition to the main components, the spectrum of minor manifestations may include ectodermal dystrophy, other endocrinopathies, such as hypergonadotripic hypogonadism, insulin-dependent diabetes, autoimmune thyroiditis, and pituitary dysfunction. Moreover, gastrointestinal disorders (chronic atrophic gastritis, pernicious anemia, malabsorption, autoimmune hepatitis and cholelithiasis), skin diseases (vitiligo and alopecia), keratoconjunctivitis, immunological defects, asplenia may be present (70). More rare manifestations of the disease include immune-mediated central and peripheral neurological manifestations, such as chronic inflammatory demyelinating polyneuropathy (54) and posterior reversible encephalopathy syndrome (PRES) (75), tubulointerstitial nephritis, autoimmune bronchiolitis, reversible metaphyseal dysplasia, hypokalemia, and hypertension (72).

The majority of APECED components have been correlated with specific autoantibodies that may represent an useful tool for the diagnosis and the follow-up of patients (**Table 1**). Autoantibodies' profile may parallel clinical expression even though a strong correlation with the phenotype and the severity of the disease is not always present. Indeed, only some autoantibodies are highly predictive of specific organ's failure, being detectable years before the onset of the overt clinical manifestations.

APCED-related CMC has been associated with the presence of specific autoantibodies against the Th17-related cytokines interleukin- (IL-) 22 and IL-17F (76, 77). A parathyroid-specific autoantigen called NACHT leucine-rich-repeat protein 5 (NALP5), which is expressed in the cytoplasm of the main cell type in the parathyroid glands (78), has been recently proposed as the immunological hallmark of APECED-related CH.

Table 1 | Clinical counter part of autoantibodies profile in APECED [modified by Capalbo et al. (73)].

Clinical features	Autoantibodies
CMC	Abs against IL-22, IL-17F, and myosin-9
ENDOCRINE MANIFESTATIONS	
HP	Abs against NALP5
AD	Abs against CYP21, CYP11A1, CYP17
Ovarian failure	Ab against CYP11A1, CYP17, and NALP5
Type 1 diabetes	Ab against IA-2 and insulin
Autoimmune thyroiditis	Ab against TPO and Tg
NON-ENDOCRINE MANIFESTATIONS	
Ectodermal manifestations	
Vitiligo	Abs against Melanocytes, SOX-9, SOX-10, and AADC
Alopecia	Abs against TH
Gastrointestinal manifestations	
Autoimmune gastritis/ pernicious anemia	Abs against parietal cells and IF
Autoimmune hepatitis	Abs against CYP-1A2, CYP-2A6, AADC, and TPH
Autoimmune enteropathy	Abs against TPH, HD, and GAD
Rare manifestations	
Pulmonary disease	Abs against KCNRG
Demyelinating polyneuropathy	Abs against myelin protein zero
Tubular interstitial nephritis	Abs against proximal tubule
Non-organ specific Abs	Abs against IFN-α and IFN-ω

Abs, autoantibodies; IL-17F, interleukin 17F; IL-22, interleukin 22; NALP5, NACHT leucine-rich-repeat protein 5; CYP21, 21-hydroxylase; CYP11A1, cholesterol side-chain cleavage enzyme; CYP17, 17-α-hydroxylase; IA-2, tyrosine phosphatase-like protein; TPO, thyroid peroxidase; Tg, thyroglobulin; AADC, aromatic l-amino acid decarboxylase; TH, tyrosine hydroxylase; IF, intrinsic factor; TPH, tryptophan hydroxylase; HD, histidine decarboxylase; GAD, glutamic acid decarboxylase; CYP-1A2, cytochrome P450 1A2; CYP-2A6, cytochrome P450 2A6; KCNRG, potassium channel-regulating protein; IFN-α, interferon α; IFN-ω, interferon ω.

Antibodies against the enzyme 21-hydroxylase (CYP21) are strongly associated and highly predictive for the development of AD in patients with CH and/or CMC (79, 80). Steroidogenic enzymes such as Cholesterol side-chain cleavage enzyme (CYP11A1) and 17α-hydroxylase/17,20-lyase (CYP17) represent a further targets of autoimmune reaction against adrenal cortex, moreover they are highly correlated with ovarian insufficiency due to lymphocytic oophoritis and can precede the clinical onset of the component (81, 82). Autoimmune gastritis is associated with the presence of autoantibodies against parietal cells and intrinsic factor (IF), the latter being involved in the development of pernicious anemia (83). The presence of autoantibodies against tryptophan hydroxylase (TPH), an enzyme involved in the synthesis of neurotransmitters in the nervous system and in the gastrointestinal endocrine cells correlates with Autoimmune enteropathy (84–87). Moreover, autoantibodies against both histidine decarboxylase (HD), an enzyme expressed in entero-chromaffin-like cells, and GAD (88, 89) have been associated with an autoimmune intestinal involvement. AH is mainly associated with the presence of autoantibodies against cytochrome P4501A2 (CYP-1A2), CYP-2A6, and aromatic L-amino acid decarboxylase (AADC), even though other types of autoantibodies, such as those directed against TPH, have been correlated with the AH component of the APECED phenotype (54, 90–92). Complement-fixing melanocyte autoantibodies and antibodies against transcription factors SOX-9, SOX-10, and AADC (83, 89) and tyrosine hydroxylase (TH) strongly correlate with the presence of vitiligo and alopecia (72, 83). Recently, several reports have confirmed an important role of autoantibodies against IFN-α and IFN-ω, which, although not tissue-specific, have been detected in the serum of almost all APECED patients (93, 94). Furthermore, they appear at a very early stage, often before the onset of any clinical manifestation. With this regard, their presence may be considered as an additional diagnostic marker of the disease, especially in those cases with an atypical presentation (94, 95). Although autoantibodies' production seems to be a key-event in the development of the clinical disease, their role in the pathogenesis of APECED still remains to be defined.

APECED is a paradigmatic example of an autoimmune monogenic disease, however, the phenotypic presentation can widely vary from one patient to another (67, 70, 96, 97). Indeed, there are observations documenting a genotype-phenotype correlation only for specific traits (98, 99), but a clear genotype-phenotype correlation is lacking. We have, recently, reported on a family with an extremely wide intra-familial clinical variability despite the same mutation of AIRE (100). These observations suggest that genetic background is not able to explain alone the variability of the clinical expression and the severity of APECED and that, as for other monogenic diseases, the phenotypic variability of the syndrome may result from the complex interaction between several genetic, epigenetic, immunological, and/or environmental factors. The HLA class I and class II alleles have been reported to confer susceptibility to develop autoimmune diseases, such as Type 1 diabetes and autoimmune thyroid diseases (101). Only few studies investigated the association between the APECED phenotype and HLA genotypes, reporting conflicting results. In fact, although some studies did not find any significant association between HLA antigens class I or II and autoantibodies' production or clinical expression of the disease (74, 102–104), other showed an increased frequency of specific HLA genotypes in APECED patients (105). However, in a more recent study on 18 Sardinian patients (54) autoimmune hepatitis, as well as LKM autoantibodies, have been found to be strongly associated with HLA-DRB1*0301/DQB1*0201. However, there is no evidence indicating that the HLA haplotype might be associated to a particular severity of the disease. Infectious agents are potent stimuli for the immune system, and thus both viruses and bacteria can be considered as trigger of an autoreaction via different mechanisms, such as molecular mimicry, bystander activation, and epitope spreading (106–111). Moreover, evidence suggests that a genetically determined susceptibility may favor the development of an autoimmune disorder after an infection. Many viruses have also been proposed as factors exacerbating several autoimmune processes (112). However, the role of the infectious

triggers has not been sufficiently investigated in patients with APECED, and preliminary results did not show any significant effect of different infections on the phenotypic expression of the syndrome (100). As already mentioned, along with the central tolerance network, which is primarily involved in the pathogenesis of APECED, several peripheral mechanisms are capable of contributing to the control and regulation of the immune system. These factors are involved in maintenance of the homeostasis by controlling residual auto-reactive clones, which escape negative selection within the thymus and play a significant role in preventing or minimizing reactivity to self-antigens. The peripheral tolerance recognizes as possible mechanisms the induction of functional anergy with inactivation of self-reactive T-cells, deletion of auto-reactive clones by apoptosis, through Fas/FasL interaction, and the suppressive action of Tregs. An additional mechanism involved in controlling reactivity to self engages in the periphery is represented by NK cell activity. A possible role of altered peripheral tolerance in the pathogenesis and clinical expression of APECED might be hypothesized also considering that recent evidence suggesting that Aire may also be implicated in the control of peripheral mechanisms dedicated to the peripheral maintenance of self-tolerance. In the periphery, Aire is expressed in DCs and a specific population of extrathymic Aire-expressing cells (113, 114). As in the thymus, also in secondary lymphoid organs Aire is required for the expression of many TSAs. However, only few studies investigated the functionality of peripheral tolerance mechanisms in patients with APECED and the role of a failure in the peripheral mechanisms of Aire's function is still poorly defined. Studies on animal models of APECED suggest that Aire does not influence *per se* Tregs as in Aire-KO mice the number of CD4+CD25+ cells are normal, and the functionality in *in vitro* suppression assays is normal as well (115, 116). However, the link between Aire and Treg cells is still not fully understood. Some recent studies suggest that Aire-expressing mTECs are involved in the generation of TSA-specific Foxp3+ Treg cells. A recent study supports this concept by showing that Aire-expressing mTECs, in addition to providing an antigen reservoir, also serve as APCs, thus enhancing the selection of Treg cells. The commitment of Tregs was shown to occur independently of Foxp3, and interaction of developing thymocytes with thymic stromal cells may drive the differentiation of a thymocyte subpopulation into the Treg cell lineage and, subsequently, trigger the expression of Foxp3 (117). Some adult APECED patients have lower proportion of Tregs (118), this finding being probably related to chronic infections, to the extent of autoimmune inflammation or therapy. Unfortunately, Tregs have been evaluated in only two children with APECED. Although in these children the number of Tregs was reduced in comparison to healthy controls, confirming the results obtained in adult patients, this reduction was not related to the severity of the disease, thus ruling out a potential role in modulating the clinical expression of the syndrome (100).

CLOSING REMARKS

Although APECED is a monogenic autoimmune disease, the great variability of the clinical expression and the absence of a clear genotype-phenotype correlation implies that, beyond AIRE mutations, other susceptibility factors such as immunological and environmental factors may be involved in the pathogenesis of the disease. The evidence of a role of an impairment of central and peripheral tolerance and of other susceptibility factors in the phenotypic variability of APECED is limited and needs to be further investigated. So far, the reason of such variability still remains obscure. Unraveling the open issues of the molecular basis of APECED, will be extremely useful in improving the diagnosis, management, and therapeutical strategies of this complex disease. As for other Mendelian diseases, total exome sequencing could be a good perspective to analyze other genetic variations and to identify potential disease-modifying genes involved in the clinical expressivity of organ-specific autoimmunity.

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Thymic stromal alterations and genetic disorders of immune system

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In this specialty section of the journal, we host a topic focused on thymic stromal alterations and genetic disorders of immune system. The thymus is a specialized organ of the immune system where, through stage-specific differentiation of hematopoietic progenitor cells, fully mature and self-tolerant T cells origin. The process is strictly dependent on the link between the thymic stromal cells (TSCs), which allow the selection of a functional and self-tolerant T-cell repertoire, and the thymus tridimensional architecture. Indeed, the interaction between the developing thymocytes and the stromal cells is crucial for the development of both T cells and TSCs (1, 2). In both human and mice, the primordial thymic epithelial cells (TECs) are yet unable to fully support the T-cell development and only after the transcriptional activation of the *Forkhead-box n1* (*FOXN1*) gene, this essential function is acquired. Most of the information concerning the T-cell development came out from studies on mice carrying null mutation in *FOXN1* gene. In humans, as detailed in the Romano et al. review, the Nude/SCID phenotype is characterized by congenital alopecia of the scalp, eyebrows, and eyelashes, nail dystrophy, and a severe T-cell immunodeficiency, inherited as an autosomal recessive disorder (3). As extensively approached in the Villa et al. review, the intercellular cross-talk is also essential to support the maturation of Foxp3C natural regulatory T cells. In Omenn syndrome (OS), caused by hypomorphic Rag alterations, an infiltration of peripheral tissues by activated T cells and immune dysregulation have been found (4). The authors discuss on abnormalities of thymic microenvironment in OS with a special focus on the defective maturation of TECs, and impairment of central tolerance.

The commonest association of thymic stromal deficiency resulting in T-cell immunodeficiency is the DiGeorge syndrome (DGS), discussed in the Davies review. In this syndrome, however, the immunological impairment is highly variable, ranging from normal to a severe immune defect in rare individuals, thus suggesting that partial thymic hypoplasia may occur or that extrathymic sites of differentiation play a role in the process (5). The difference in the immunological defects between DGS and the Nude/SCID phenotypes implies that FOXN1 controlled genes are mandatory for a fully mature T-cell development process rather than the integrity of the thymus itself.

It is known that autoimmune regulator (*AIRE*) gene plays a central role in the induction of central tolerance, and different mechanisms of action have been hypothesized for this process. According to the most reliable theory, AIRE directly induces the production of tissue-specific antigens (TSA) (6). However, recent evidence suggests that another mechanism for negative selection of self-reactive thymocytes may be due to AIRE-induced differentiation of medullary TECs, and regulation of the expression of intrathymic chemokines directed to antigens presenting cells (APCs), such as thymocytes and dendritic cells (7). In their reviews, Laan and Peterson and Kisand et al. give an overview on what is known about the different mechanisms through which AIRE induces central tolerance.

The process aimed at the elimination of potential self-reactive T cells in the thymus is crucial for preventing the onset of autoimmune diseases. As discussed in the Akiyama et al. paper, medullary epithelial cells play a central role in the process through the regulation of gene expression, and, in particular, of those genes encoding for the TNF family cytokines, RANK ligand, CD40 ligand, and lymphotoxin. These genes promote the differentiation of AIRE- and TSA-expressing mTECs (8).

The mechanism by which a single *AIRE* gene can influence the transcription of such a large number of TSA within mTECs has been discussed in the Matsumoto et al. paper. Two models have been proposed. The first one implies a direct transcriptional control of *AIRE* on TSA, while the second one is based on the role of *AIRE* on the maturation program of mTECs (9).

The clinical and immunological phenotype of patients affected with autoimmune polyendocrinopathy ectodermal dystrophy (APECED), as reviewed by Petteri Arstila and Jarva, is characterized by multiple endocrine deficiencies, the most common manifestations being hypoparathyroidism, Addison's disease, hypogonadism, and secondary amenorrhea, usually associated with the presence of autoantibodies toward the target tissues (10). However, the phenotype and, therefore, the underlying pathogenic mechanism, are even more complex, in that Chronic Mucocutaneous Candidiasis is also a prominent part of the disease. This clinical entity is related to abnormalities in the Th17-related cytokines, which are mostly involved in immune defenses against Candida (11). Finally, high titers of neutralizing autoantibodies against type I interferons, which have been shown to downregulate the expression of interferon-controlled genes, have been documented (12).

In this Research Topic, De Martino et al. focus their attention on the complexity of the APECED phenotype in that a wide variability of the clinical expression, in the presence of the same genotype alteration, has been found (13). They suggest that additional

mechanisms, in addition to AIRE function, are involved in the pathogenesis of the disease. This might be helpful to understand not only the molecular basis of APECED but will also help improve diagnosis, management, and therapeutic strategies to treat this complex disease.

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CHAPTER IV

Targeted next generation sequencing as an approach to patients with severe forms of congenital immunodeficiencies

Primary immunodeficiency disorders (PIDs) represent a heterogeneous group of inherited disorders characterized by poor or absent function in one or more components of the immune system, that result in chronic, recurrent and life-threatning infections if not promptly diagnosed and treated (2). Traditionally, PIDs are classified according to the component of the immune system that is primarily disrupted: innate or adaptive immunity, the latter comprising antibody deficiencies and combined immunodeficiencies. In the last 20 years, thanks to the progress in molecular technologies, a remarkable improvement of the knowledge in the field of PIDs, concerning both their pathogenetic mechanisms and clinicalfeatures, has been observed.

Nowadays, about 300 forms of well-characterized PIDs have been identified underlying complex phenotypes which encompass a wide spectrum of clinical features ranging from recurrent bacterial infections to other unusual manifestations,such as autoimmune disorders, cancer susceptibility, allergy and autoinflammation. In many cases, children affected with novel forms of PIDs also show extrahematopoietic alterations, adding to the complexity of the phenotype (129). In the more severe forms, as severe combined immunodeficiencies (SCIDs), bacterial, viral, and fungal infections are life-threatening conditions (130). With this regard, a timely diagnosis may lead to a prompt and appropriate treatment to

reconstitute their immune system. Moreover, the early diagnosis of PID is desirable for preventing significant disease-associated morbidity and mortality.

The overall prevalence of the severe forms of PIDs in the general population is approximately 1:10.000 live birth. However, a recent study reported that PIDs are underdiagnosed and, therefore, they are presumably more common than previously estimated. Thus far, the diagnosis of a specific PID has been based on the demonstration of a functional immune defect and on the subsequent identification of candidate genes, which are selectively involved in the biochemical pathway implicated in that specific functional alteration.

The Sanger sequencing of candidate genes is a costly and time consuming procedure, and, not infrequently, unsuccessful. This approach certainly leads to a considerable delay between the onset of symptoms and the time of diagnosis, as usually reported. However, it should be emphasized that in most cases, the identification of the underlying genetic alteration is not possible.

Moreover, a prompt and accurate diagnosis of PID with this traditional approach may be difficult also because of the variety of clinical/immunological symptoms. Clinical heterogeneity as different mutations in the same gene can lead to different clinical phenotypes. For example, a specific recombination-activating gene 1 (RAG1) variant encoding for a protein with partial recombinant activity might produce Omenn syndrome, a leaky Severe Combined Immunodeficiency or a Common Variable Immunodeficiency phenotype. Genetic heterogeneity implies that the selection of candidate genes to screen based on the individual patient's clinical and immunological features may not be easy in the majority of cases, since several genes may be implicated in a specific phenotype. In addition, different clinical presentations are attributed to a single gene defect, reflecting not only the variety of

mutations within each gene but also the interference of modifying genetic factors that may influence the phenotype, as observed between individuals with the same mutation. Finally, it should be outlined that not all the PIDs causing genes are known.

More recently, advances in next generation DNA sequencing allowed new gene identification through whole exome or whole genome sequencing (WES, WGS) of several forms of PIDs of unknown causes making the genetic identification of immunodeficiency syndromes more efficient (9). Only in the last two years, using this technology 34 new gene defects have been identified. Most of these immunodeficiencies are rare, even though some of them occur more frequently than what previously reported, as documented by several groups (10). Based on the principle of massively parallel sequencing, NGS technology provides an advanced tool to dramatically increase the speed at which DNA can be sequenced at a lower cost as compared to the traditional Sanger sequencing approach.

4.1 Targeted next generation sequencing revealed MYD88 deficiency in a child with chronic Yersiniosis and granulomatous lymphadenitis

Myeloid differentiation factor (MyD)88 is a key downstream adapter for most Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) (131). Its pathway regulates the synthesis of several inflammatory cytokines such as IL-1β, IL-6, IL-8, interferon (IFN)-α/β, IFN-γ and tumor necrosis factor (TNF)-α (132). *MYD88* deficiency is a very rare, autosomal recessive primary immunodeficiency, associated with life-threatening and recurrent pyogenic bacterial infections, including invasive pneumococcal disease (133). In *MYD88*-deficient patients, impaired polymorphonuclear neutrophil mobilization and/or frank neutropenia may occur from the onset of infection, presumably related to the lack of IL-8 production. Gastrointestinal infections are rare (134). Moreover, TLRs play a key role in the immunological response to flagellated Gram-negative bacterium (135). Of note, Yersinia enterocolitica is able todeactivate TLR-induced signaling pathways, by cleaving Myd88, triggering to apoptosis in macrophages (136). This mechanism may increase Yersinia virulence also inimmunocompetent hosts. In a recent paper by von Bernuth et al, MyD88-deficient mice resulted susceptible to almost nearly all the microbes tested, including bacteria, viruses, protozoa and fungi, highlighting the importance of TLR in the immuneresponse to different pathogens. By contrast, patients with MyD88- or IRAK-4-deficiencies have been shown to be susceptible to invasive and non-invasive infections with only a few Gram-positive and Gram-negative bacteria, maybe as a consequence of the redundancy of the immune system (137). However, the predominance of Gram-positive bacteria in patients with MyD88 and IRAK-4 deficiencies may result, at least in part, from a limited exposition to the different antigens in certain geographic areas. The evidence of

chronic Yersiniosis in this patients, expand the variety of organisms, which can lead to infections in subject affected with this syndrome.

We recently reported the case of a 2-year-old female born to consanguineous parents of Rom ethnia, affected with chronic Yersiniosis, recurrent granulomatous lymphadenitis, and episodicneutropenia in the absence of recurrent invasive pneumococcal disease. Through a targeted next generation sequencing panel, including genes responsible for immunodeficiency, we found a homozygous in-frame deletion (p.Glu66del) in the Myd88 gene, that allowed us to perform a diagnosis in this patient.

These data have been published as *Letter to the Editor* on *Journal of Allergy and Clinical Immunology*, for the manuscript see below.

Letter to the Editor

Targeted next-generation sequencing revealed *MYD88* deficiency in a child with chronic yersiniosis and granulomatous lymphadenitis

To the Editor:

Yersiniosis is a food-borne illness, usually self-limited. Severe clinical courses may occur in chronic conditions, particularly in immunocompromised individuals.[1]

Here, we report on the case of a 2-year-old girl born to consanguineous parents of Roma descent (a traditionally itinerant ethnic group living mostly in Europe and the Americas, who originate from Northern India), with chronic yersiniosis, recurrent granulomatous lymphadenitis, and episodicneutropenia. Using a targeted next-generation sequencing panel for immunodeficiency genes, a homozygous in-frame deletion in the *MYD88* gene was found. MyD88 is a key downstream adapter for most Toll-like receptors (TLRs) and IL-1 receptors (IL-1Rs).[2] *MYD88* deficiency has been associated with life-threatening and recurrent pyogenic bacterial infections, including invasive pneumococcal disease.[3,4]

The child was born at term from a pregnancy complicated by maternal syphilis, which required a postnatal treatment with benzathine penicillin. During the treatment, she developed a severe neutropenia, from which she spontaneously recovered. A congenital syphilis infection was excluded by serological, blood culture, and cerebrospinal fluid examination, cerebral ultrasound, and long bone x-ray image. Familial history was significant for 3 brothers who died in childhood of infectious diseases, 2 of meningitidis and sinusitis and 1 of enteritis (Fig 1, *A*). No further information was available about the brothers' clinical history. At the age of 7 months, she developed an episode of upper left arm edema associated with fever followed by an intestinal occlusion. Exploratory laparotomy revealed severe stenosis of the ileal tract from 10 to 40 cm proximal to the ileocecal valve, associated with mesenteric adenitis. Intestinal wall histology revealed conspicuous infiltration of bacilli (Fig 2, *C* and *D*), vast areas of marginal necrosis, full-thickness massive edema, and inflammatory infiltrates (Fig 2, *E* and *F*). Cultures of stool and of the bowel wall were negative for bacteria, parasites, and mycobacterium tuberculosis. Cytomegalovirus, human immunodeficiency virus (HIV1/HIV2), Salmonella/Brucella, and *Treponema pallidum* (Rapid plasma reagin/treponema pallidum hemagglutination assay, *T pallidum* IgM/IgG) were also excluded by serological examinations. At the age of 13 months, she developed a new episode characterized by fever, abdominal pain, diarrhea, and vomiting. On this occasion, blood culture grew *Yersinia*, which required treatment with amoxicilline/clavulanic acid.

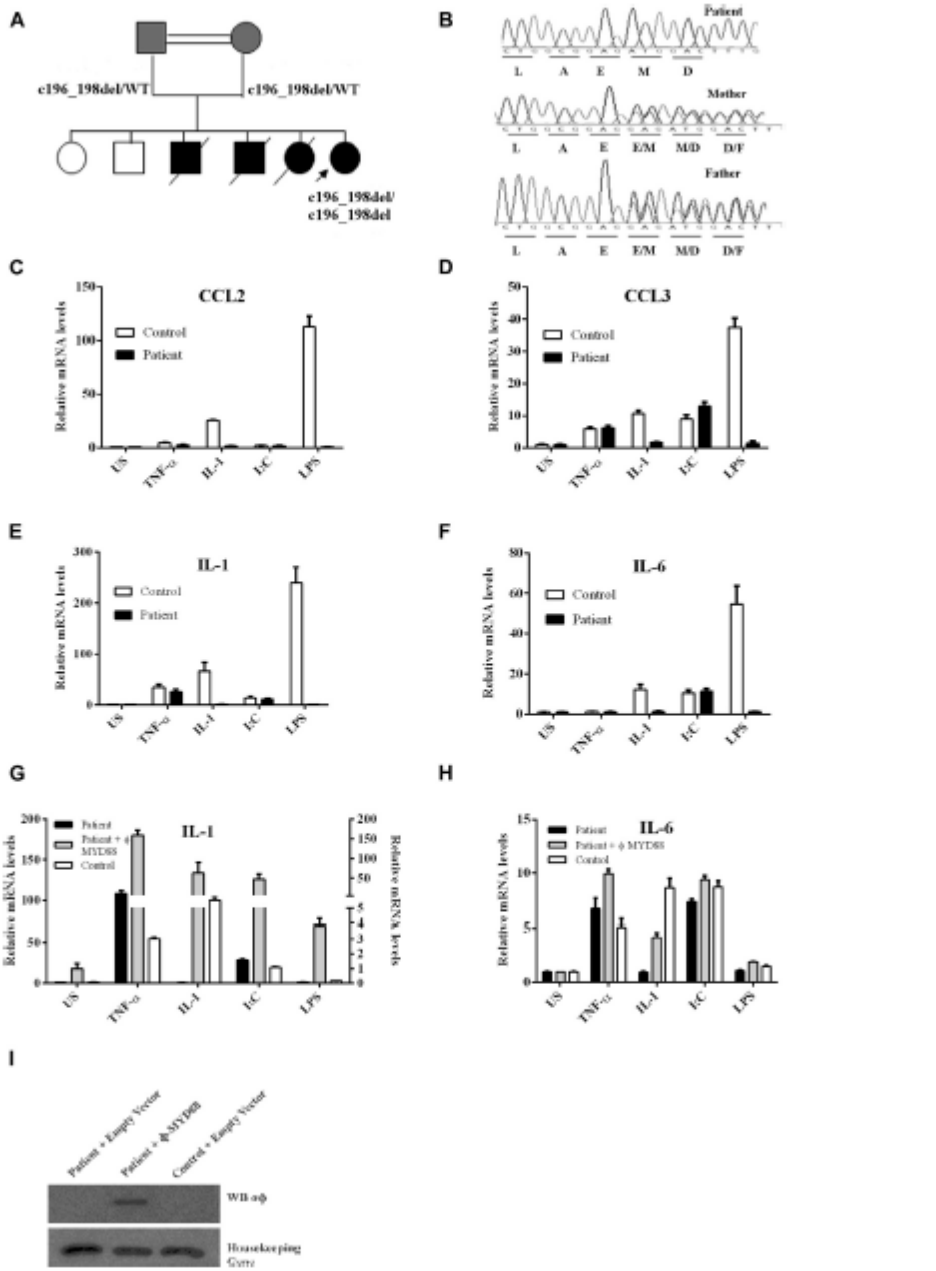
At the age of 8 months, she developed axillary (Fig 2, *A* and *B*) and epitrochlear suppurative lymphadenitis of the left arm. The histological examination of the 5-cm diameter cold axillary colliquative lymphadenitis revealed a mixed inflammatory reaction characterized by T and B lymphocytes, plasma cells, and eosinophils. Histiocytes, mixed with multinucleated giant cells arranged in a palisading pattern, were observed, consistent with a granulomatous inflammatory process. Cultures from the purulent exudate grew *Staphylococcus aureus* sensitive to amoxicilline/clavulanic acid. The patient never experienced eczema or any other cutaneous lesion, and abscesses developed on an otherwise healthy skin. Laboratory examinations revealed microcytic hypocromic anemia (hemoglobin, 8.9 g/dL; mean corpuscular volume, 60 fl; mean corpuscular hemoglobin, 18 pg), eosinophilia (750 cells/μL), and normal inflammatory index (C-reactive protein, 0.1 mg/dL; ferritine, 73.8 ng/mL). After unsuccessful intravenous antibiotic therapy, surgical drainage was required. Prophylactic treatment with cotrimoxazole and amoxicilline/clavulanic acid was then given for potential recurrence of *S aureus* lymphadenitis.

An immunodeficiency was suspected on the basis of the clinical features and familial history. Other genetic disorders, not directly involving the immune system, were ruled out by the clinical history and routine laboratory examinations. First-level immunological examinations revealed normal IgG, IgM, and IgA levels, increased IgE levels (1190 IU/mL), normal levels of standard lymphocyte subpopulations ($CD3^+$ 57%, $CD4^+$ 41%, $CD8^+$ 12%, $CD56^+$ 14%, $CD19^+$ 19%, $CD3^+HLA^-DR$ 3%) (for absolute numbers, see Table E1 in this article's Online Repository at www.jacionline.org) and of adhesion molecules (absolute monocyte number was 440 cells/μL and CD11a/CD18, CD11b/CD18, and CD11c/CD18 on the leucocyte gate was 99%). Specific antibodies were detectable, and the proliferative response to mitogens was normal. The study of the response to Prevenar13 revealed an impaired IgM, but not IgA and IgG, response against pneumococcal polysaccharides, as previously reported by Maglione et al.[5] This finding, associated with the evidence of a low titer of isohemagglutinins, suggests an impaired response against T-independent polysaccharidic antigens (see Table E2 in this article's Online Repository at www.jacionline.org). Nitroblue tetrazolium chloride and dihydrorhodamine 123 assays ruled out the chronic granulomatous disease. AT+B+NK+ combined immunodeficiency due to hypomorphic *RAG1/2* mutation, consistent with the presence of granulomatous inflammation of skin, was excluded by molecular analysis. Hyper-IgE syndrome score was ruled out through the molecular analysis of *STAT3*.

At the age of 11 months, she again developed a severe neutropenia, which eventually required G-CSF treatment. Congenital neutropenia was ruled out with negative *ELANE* gene sequencing.

After written informed consent from the parents for molecular genetic studies, a targeted next-generation sequencing panel (TaGSCANv.2: Targeted Gene Sequencing and Custom Analysis), including 572 genes and customized for 55 immunodeficiency genes, was run (see the Methods section and Table E3 in this article's Online Repository at www.jacionline.org).[6] A homozygous known pathogenic in-frame deletion, c.196_198del GGA (p.Glu66del; also referred to as E65del and E52del[3,7,8]), was identified in exon 1 of the *MYD88* gene. This variant has been previously reported in several affected families, at least 2 of which were of Rom descent with consanguineous parents. This deletion removes a single conserved glutamic acid residue in the Death domain, resulting in greatly diminished protein levels.[7] Sanger sequencing was used to verify the finding and confirm carrier status of both parents (Fig 1, *A* and *B*).

We studied the effect of MYD88 deficiency in TLR and IL-1R responses of PBMCs from the patient. *Il-6*, *il-1*, *ccl2*, and *ccl3* mRNA induction following TLR4 or IL-1R agonists


A
c196_198del/WT
c196_198del/WT
c196_198del/
c196_198del
B
Patient
L A E M D
Mother
L A E E/M M/D D/F
Father
L A E E/M M/D D/F
C
CCL2
Control
Patient
Relative mRNA levels
US
TNF-α
IL-1
I:C
LPS
D
CCL3
Control
Patient
Relative mRNA levels
US
TNF-α
IL-1
I:C
LPS
E
IL-1
Control
Patient
Relative mRNA levels
US
TNF-α
IL-1
I:C
LPS
F
IL-6
Control
Patient
Relative mRNA levels
US
TNF-α
IL-1
I:C
LPS
G
IL-1
Patient
Patient + ϕ MYD88
Control
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Relative mRNA levels
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TNF-α
IL-1
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IL-6
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Patient + ϕ MYD88
Control
Relative mRNA levels
US
TNF-α
IL-1
I:C
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I
Patient + Empty Vector
Patient + ϕ-MYD88
Control + Empty Vector
WB α-ϕ
Housekeeping
Gene

was abolished, while they were normally induced after TNF-α and TLR3 stimulation (Fig 1, *C-F*). Fibroblasts from the MYD88-deficient child and a healthy control were transiently transfected with an expression vector encoding the wild-type *MYD88* gene. Cells from MYD88-deficient patient regained IL-1β and LPS responsiveness after transfection with the wild-type *MYD88* gene, as shown by the levels of IL-1 and IL6 production (Fig 1, *G* and *H*) (see the Methods section).

In *MYD88*-deficient patients, impaired polymorphonuclear neutrophil mobilization and/or frank neutropenia may occur from the onset of infection, presumably related to the lack of IL-8 production. Gastrointestinal infections are rare.[9] Moreover, TLRs play a key role in the immunological response to flagellated Gram-negative bacterium, thus explaining why the patient developed chronic yersiniosis.[10] Of note, *Yersinia enterocolitica* is able to deactivate TLR-induced signaling pathways, by cleaving Myd88, triggering apoptosis in macrophages.[11] This mechanism may increase Yersinia virulence in immunocompetent hosts also. In a recent article by von Bernuth et al,[12] MyD88-deficient mice were found to be susceptible to almost nearly all the microbes tested, including bacteria, viruses, protozoa, and fungi, highlighting the importance of TLR in the immune response to different pathogens. In contrast, patients with MyD88 or IRAK-4 deficiencies have been shown to be susceptible to invasive and noninvasive infections with only a few Gram-positive and Gram-negative bacteria, maybe as a consequence of the redundancy of the immune system. However, the predominance of Gram-positive bacteria in patients with MyD88 and IRAK-4 deficiencies may result, at least in part, from a limited exposition to the different antigens in certain geographic areas. The evidence of chronic yersiniosis in this patient expands the list of organisms that can lead to infections in subjects affected with this syndrome. Even though we were not able to grow *Yersinia* from the bowel wall, it was suspected as being responsible for the terminal ileitis and mesenteric adenitis on the basis of the histological staining indicative of a bacillus infection.

In this study, we reported on a patient affected with chronic yersiniosis, recurrent granulomatous lymphadenitis, and neutropenia associated with an homozygous p.Glu66del variant in *MYD88*. This finding was unexpected because Myd88 deficiency is characterized by high susceptibility to invasive bacterial disease caused by *Streptococcus pneumoniae* and *S aureus*, with invasive pneumococcal disease being the hallmark of the syndrome.[9] Noninvasive bacterial diseases caused by *S aureus* or *Pseudomonas aeruginosa* have also been described.[9] The atypical presentation of our patient, characterized by chronic yersiniosis and recurrent neutropenia in the absence of recurrent invasive pneumococcal disease, made the diagnosis difficult. In these contexts, targeted next-generation sequencing may represent a rapid and low-cost tool to achieve an early diagnosis.

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FIG 1. *MYD88* deficiency impairs TLR and IL-1R responses of PBMCs. **A**, Family pedigree. The proband is indicated with an *arrow*. Healthy persons are shown in white; deceased siblings are indicated by *line crossing*. **B**, Genomic sequence analysis of *MYD88* gene showing a homozygous in-frame deletion, c.196_198del GGA (p.Glu66del). Both parents are heterozygous for the same mutation. **C-F**, Real-time PCR analysis of the mRNA extracted from the patient's PBMCs stimulated with LPS, IL-1, TNF-α, and poly (I:C) (TLR3 ligand) revealed reduced levels of IL-6, IL-1, CCL2, and CCL3, as compared with the healthy control, in response to LPS and IL-1 stimulation, while they were normally induced after TNF-α and TLR3 stimulation. **G** and **H**, Fibroblasts from a healthy control and from the MYD88-deficient child, transiently transfected with an expression vector encoding wild-type (WT) MYD88. Cells from MyD88-deficient patient regained IL-1β and LPS responsiveness after transfection with the WT *MYD88* gene, as shown by the levels of IL-1 and IL6. Y-axis values, on the *right side*, are referred to LPS stimulation. **I**, Western blotting using anti–FLAG-specific antibody to verify the transfection of patients' fibroblasts with plasmids carrying WT *MYD88*.

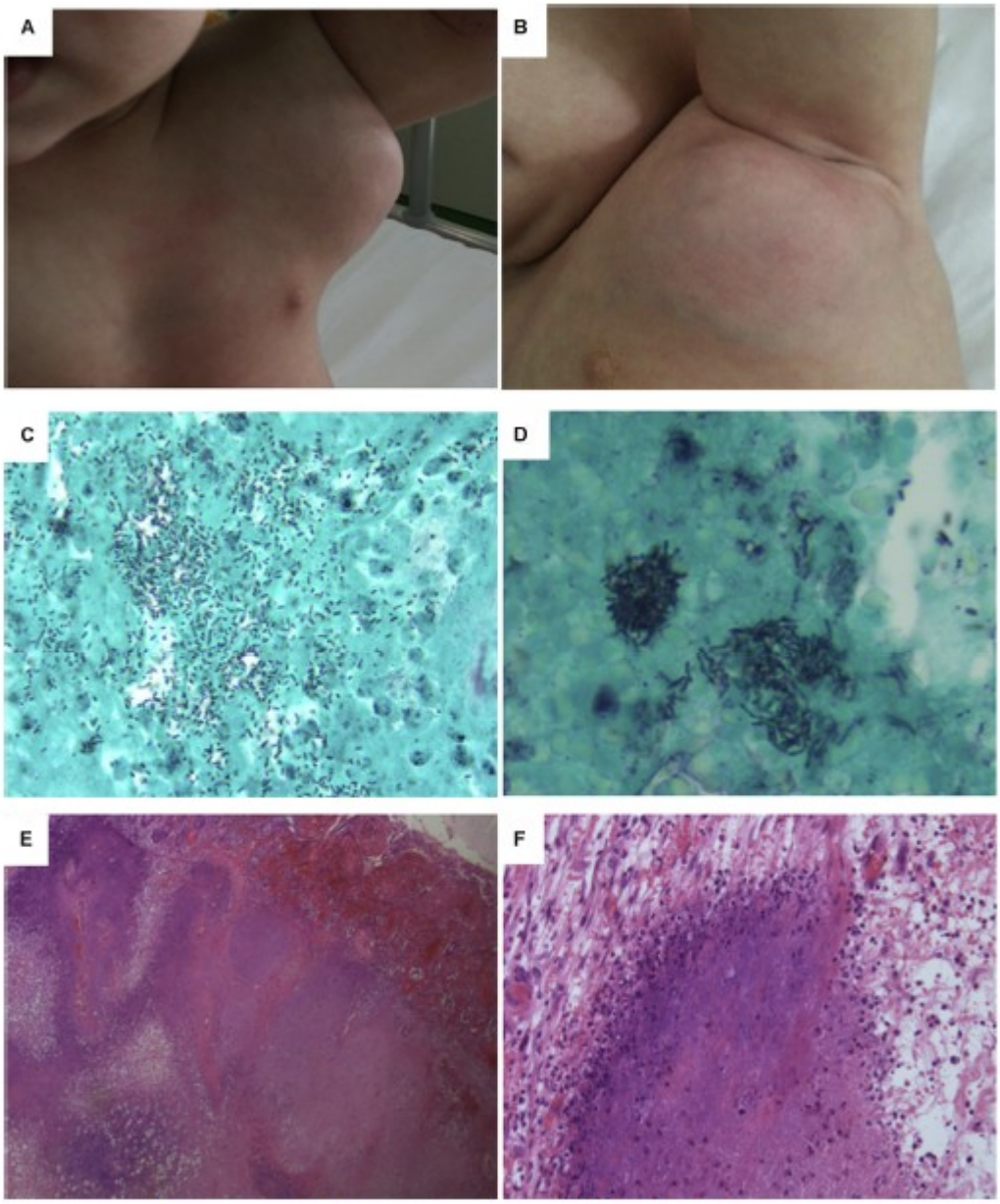

FIG 2. Clinical features. **A** and **B**, Axillary suppurative lymphadenitis of the left arm. **C** and **D**, Grocott stain revealed conspicuous infiltration of bacilli in the intestinal wall. **E** and **F**, Intestinal wall histology revealed vast areas of marginal necrosis, full-thickness massive edema, and inflammatory infiltrates.

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METHODS

Targeted next-generation panel, "TaGSCANv.2" (Targeted Gene Sequencing and Custom Analysis) was run, which includes 572 genes. Briefly, samples were prepared for sequencing using TruSight Rapid Capture with TruSight Inherited Disease Oligos (Illumina, San Diego, Calif); the MT genome is covered at 1000× by the addition of MT oligos during enrichment (Integrated DNA Technologies, Coralville, Iowa). Samples were sequenced to at least 2.5Gb on an Illumina MiSeq with TruSeq v3 reagents, yielding paired 250 nucleotide reads. Alignment, variant calling, and analysis were performed as previously described.[E1] In this case, analysis was customized for immunodeficiency, which limited the genes examined to 55 (see Table E3). Variant analysis was confined to coding and splice variants with a minor allele frequency of 1% or less in the Children's Mercy Hospital internal database. Two variants remained after filtering. One variant, in the *FERMT3* gene associated with leukocyte adhesion deficiency type III, was not pursued because the minor allele frequency is 0.96 in European Americans (National Heart, Lung, and Blood Institute database). No second variant was identified, and there was limited phenotypic overlap. In addition, a homozygous known pathogenic in-frame deletion, c.196_198del GGA (p.Glu66del; also referred to as E65del and E52del in the literature[E2–E4]), was identified in exon 1 of the *MYD88* gene. This variant has been previously reported in several affected families, at least 2 of which were of Roma descent with consanguineous parents. This deletion removes a single conserved glutamic acid residue in the Death domain, resulting in greatly diminished protein levels.[E4] Sanger sequencing was used to verify the finding and confirm carrier status of both parents (Fig 1, *A* and *B* in the text).

PBMCs were isolated from patients by density gradient centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany). For the cytokine mRNA production, PBMCs were cultured in RPMI 1640 supplemented with 1% FBS, for 24 hours without stimulation and then stimulated with LPS 1 ng/mL, IL-1β 10 ng/mL, TNF-α 20 ng/mL, or poly(I:C) (TLR3 ligand) 25 μg/mL. The mRNA, extracted from the PBMCs, was analyzed by real-time PCR and standard procedures.

For the complementation of patients' fibroblasts with plasmids carrying wild-type MYD88, fibroblasts were incubated with a mixture containing Lipofectamine 2000 reagent (Invitrogen #52887) and plasmids, as previously reported.[E2] This complex was then removed and fibroblasts were incubated for 18 hours in Dulbecco modified Eagle medium supplemented with 10% FBS before stimulation. Cytokine mRNA production was assessed in fibroblasts cultured in Dulbecco modified Eagle medium supplemented with 10% FBS, incubated for 48 hours without stimulation and then with TNF-α, IL-1β, or poly(I:C) for 6 hours.

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TABLE E1. Absolute number of cell subpopulation

Cell subpopulation	Absolute number (reference value)
Leukocytes	8,960 (6,700-14,000)*
Lymphocytes	5,320 (1,800-18,700)
T cells ($CD3^+$)	3,032 (1,400-11,500)
CD4 cells	2,181 (1,000-7,200)
CD8 cells	638 (200-5,400)
CD3 HLA-DR	159
B cells ($CD19^+$)	1,010 (130-6,300)
Natural killer cells ($CD56^+CD3^-$)	745 (68-3,900)

NA, Not available.
*Normal reference value.[85]

TABLE E2. Immunologic humoral evaluations of the patient

Laboratory evaluation	
Immunoglobulins (mg/dL)	
IgG	1180 (351-919)*
IgA	104 (10-85)
IgM	88 (38-204)
IgE (KU/L)	1190
Specific antibodies	
HBV antigen antibodies (mIU/mL)	143.9
HSV (IgG/IgM)	+/−
VZV (IgG/IgM)	+/−
CMV (IgG/IgM)	−/−
Rosolia (IgG/IgM)	+/−
Antibody response to pneumococcal polysaccharides (IgG/IgA/IgM)†	
IgG	8.0
IgA	27.2
IgM	0.7
Isohemagglutinins	Anti-A 1:32 Anti-B 1:4

CMV, Cytomegalovirus; *HBV*, hepatitis B virus; *HSV*, herpes simplex virus; *VZV*, varicella zoster virus.
*Normal reference values.
†Data indicate the fold-increase in the antibody titer after the booster. A positive response is defined as a threefold increase in the antibody titer.

TABLE E3. List of the 55 studied genes

ADA
AP3B1
ATM
BLM
BLOC1S6
BTK
CD19
CD247
CD3D
CD3E
CD3G
CD40LG
CFP
DCLRE1C
DOCK8
ERCC2
ERCC3
FERMT3
FOXN1
FOXP3
G6PC3
GTF2H5
ICOS
IFNGR1
IFNGR2
IKBKG
IL12B
IL12RB1
IL1RN
IL2RG
JAK3
LIG4
LYST
MPV17
MYD88
NHEJ1
ORAI1
PRF1
RAB27A
RAG1
RAG2
RMRP
SH2D1A
SLC35C1
SP110
STAT1
STIM1
STX11

4.2 Diagnostics of Primary Immunodeficiencies through targeted Next Generation Sequencing

Next Generation Sequencing (NGS) technology represent a powerful, cost-effective, first-line diagnostic tool for a rapid detection of a genetic alteration, particularly in complex cases and represent a valuable and rapid alternative to the whole genome sequencing (138).

In this study we used NGS sequencing panel, including 571 targeted genes and WES to study a cohort of 45 patients affected with complex clinical phenotypes, highly suggestive for primary immunological defect, in which a diagnosis was not obtained through current diagnostic procedures. We enrolled patients who presented one or more clinical criteria (such as opportunistic infections, granuloma, chronic mucocutaneous candidiasis, intractable diarrhea, bronchiectasis, severe autoimmunity, variably associated to syndromic features and/or familiarity for similar phenotype) associated to one or more immunological alteration (such as abnormal lymphocyte subsets (absolute count < 2 SD of normal values according to ESID criteria), proliferative response to mitogens <10% of the CTR, absent/poor specific antibody response, hypogammaglobulinemia, high IgE levels (>2000 KU/l), absent cytolytic activity, alteration of class switch recombination (CSR) with or without hyper-IgM.

In four patients we found alterations of genes responsible for already genetically defined PIDs associated with unusual clinical phenotypes, confirmed at functional and molecular level (Table 2). In 8 patients we identified a total 22 gene variants in genes implicated in the immune response but not previously associated to any known PID that will be further investigated to find a correlation with the immunological and clinical features (Table 3). In 1 patient, NGS revealed multiple

genetic variants, that were consistent only with some features of the clinical phenotype (Table 4). In 10 patients, with a complex disorder not classified in any known syndrome, we found a number of genetic variants not proved as relevant for the pathogenesis. Eventually, in the remaining 23 patient NGS, including 9 T-NGS and 14 WES, did not reveal any variant at all (Table 5).

The genetic alterations previously mentioned have been divided into 4 categories: I) genetic alterations associated to a canonical PID phenotype; II) novel or already described alterations of genes causing previously defined PIDs associated with novel clinical features; III) functionally relevant genetic alterations partially consistent with novel clinical phenotypes; IV) multiple genetic variants not consistent with functional alterations individually, in whom the link between the genotype and that new phenotype is still missing. Thanks to this novel diagnostic approach, a defined diagnosis of PID was achieved in a timely manner, in 8 out of 45 subjects.

NGS technology represents a cost-effective and rapid first-line genetic approach for the evaluation of complex cases of PIDs. The advantage of this technique is the simultaneous sequencing of a panel of genes, which might allow clinicians to rapidly identify an affected gene, that, probably, would be never sought using the traditional approach based on a functional driven hypothesis. Prompt diagnosis may allow physicians to get started with the more appropriate treatment, which may often be life-saving.

Gene	Mutation	Zigosity	Inheritance	Clinical and immunological phenotype
CD40L	c.373C>T	Hom	XL	Severe hypogammaglobulinemia with hyper IgM, neutropenia, P. carinii pneumonia, CMV infection, intractable diarrhea
STAT1	c.847T>A	Het	AD	Chronic mucocutaneous candidiasis, recurrent pneumonia, hypothyroidism, lymphopenia, poor vaccine response
BTK	c.1105C>T	Hom	XL	Agammaglobulinemia
JAK3	c.856C>T	Hom	AR	T-B+NK- SCID, chronic diarrhea, pathological proliferative response to mytogens , IgA deficiency

Table 2. Genetic variants associated to typical PID

Gene	Mutation	Zigosity	Inheritance	Clinical phenotype
MYD88	c.192_194del	Hom	AR	Chronic yersiniosis and terminal ileitis, recurrent severe cutaneous granulomatous abscesses, hyper IgE, hypereosinophilia, neutropenia
PLDN	c.232C>T	Hom	AR	Partial oculocutaneus albinism, nystagmus, recurrent cutaneous infections, thrombocytopenia, leucopenia, NK deficiency
DOCK8 /CLEC7 A	c.3193delA	Hom/Hom	AR	Intractable diarrhea, eczema, malignancies (?), food allergies, hyper IgE, lymphopenia
CASP10	c.683C>T	Het	AD	Acute lymphoblastic leukemia treated with allogenic HSCT, ethmoiditis, recurrent lymphoadenopathy, autoimmune cytopenia, arthritis, hypogammaglobulinemia, hyperIgM, IgA deficiency

Table 3. Genetic variants associated to novel presentation of PID

Gene	Mutation	Zigosity	Major clinical features
CASP10	c.1202_1208del	Het	Alopecia universalis, hyperthyrotropinemia, type I diabetes mellitus, dental enamel hypoplasia, developmental delay, short stature, candidiasis, hepatomegaly, multiple skeletal abnormalities, myopia, dysmorphic features, microcephaly
DOCK8 *TRL3*	c.1907A>G c.2672A>G	Het	Inflammatory bowel disease, short stature, aspergillosis, EBV infection, low CD4+ lymphocyte subset , increased CD4 CD8 double negative T cells , normal antibody response
ADA *ERCC6*	c.377C>A c.1047A>G c.3262A>G c.2697G>A	Het	T-B+NK+ SCID treated with bone marrow transplantation, Pneumocystis carinii pneumonia, recurrent otitis, absent ossicular bone with hypoacusis of the right ear, mild brain and cerebellar atrophy, speech delay, idiopathic scoliosis
AP3B1 *PRF* *ADAMTS13*	c.787G>T c.695G>A c.272C>T c.2701G>T	Het	Interstitial lung disease CMV infection,esophageal candidiasis, strabismus, abnormal expression of perforin in NK cells, reduction of CD4+ lymphocyte subset with increase of CD19+ and HLA-DR, normal proliferative response to mytogens, normal antibody response
CFTR	c.2991G>C	Het	Hypogammaglobulinemia late onset, recurrent pneumonia, bronchiectasis, chronic sinusitis, cervical and mediastinum lymphoadenopathy, recurrent abdominal pain, hepatomegaly with low grade steatosis, splenomegaly

Table 4. Genetic variants that are not causative of PID, with possible impact on the disease

Gene	Mutation	Zigosity	Major clinical features
SYCE2	c.577G>A	Het	t(11;18)MLT1-AP12 gastric maltoma HP +, persistent oral candidiasis, sinusitis; lung cystis, chronic cough, recurrent fever, hypereosinophilia, reccurent itch, reccurent myofasciitis, hyper IgM, altered somatic hypermutation, absent CD19+ CD20- IgG+ (mature), low CD19+ CD27+ IgM+ (memory), absent CD19+ CD27+ IgM-(switched memory)
LYST	c.10235G>A		
ATR	c.5257A>G	Het	
ARSA	c.869G>A	Het	
CASP10	c.683C>T	Het	Severe aplastic anemia, hepatomegaly, Legionella p. and Aspergillus recurrent pneumonia, metacarpal deforming alterations with bone demineralization, abnormal lymphocyte proliferation, dilated cardiomyopathy, early retinophaty
IKBIKG	c.1165C>T	Het	
MEFV	c.460T>C	Het	
SP110	c.1114C>T	Het	
UNC13D	c.335G>C	Het	
ATRX	c.2247_2249del	Het	Autoimmune adrenal insufficiency, autoimmune thyroiditis, lymphadenopathy, autoimmune thrombocytopenia and neutropenia
	c.2133_2135del	Hom	
MYD88	c.10_28del	Het	
DOCK8	c.2920C>A	Het	
	c.3016C>A	Het	
	c.3220C>A	Het	
TYK2	c.3488A>G	Het	Hypogammaglobulinemia, familial IgA deficiency, hyper IgE, multiple bronchiectasis, candidiasis
TLR3	c.634-10C>A	Het	Familial IgA deficiency, multiple bronchiectasis, recurrent respiratory infections, low IgM levels
CASP10	c.1094A>C	Het	Mild hypogammaglobulinemia, undetectable CD16+ lymphocyte levels, pervasive developmental disorder
ERCC5		Het	
GJC2		Het	
CD3-ZETA	c.301C>T	Het	Hypogammaglobulinemia, recurrent pneumonia, previous alopecia, behavioral disorders, oropharyngeal candidiasis
OCRL			

Table 5. Genetic variants that are not causative of PID with undetermined impact on the disease

TECHNOLOGIES

Cells and cell cultures

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Lymphoblastoid B-cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMCs using standard procedures, and were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, California), 2 mmol L-glutamine (Gibco, Carlsbad, California), 50 g/ml, gentamycine (Gibco, Carlsbad, California), 10% Penicillin-Streptomycin (Lonza, Verviers, Belgium), and cultured at 37°C, 5% CO2. Serum starvation was induced incubating the cells in medium supplemented with 2 mmol/L L-glutammine, without FBS, for 2h.

Cord blood (CB) from the FOXN1$^{-/-}$ fetus was obtained by cordocentesis at 16 weeks of gestation. Age-matched CB cells from the CEINGE bank of Naples were used as control. Fetus parents provided written informed consent.

Leukemia cells were obtained from the bone marrow of newly diagnosed patients, with high leukemia involvement (85-100%), affected by Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), B-precursor Acute Lymphoblastic Leukemia (B-pre ALL) and T-ALL, by using Ficoll-Hypaque (Biochrom) density gradient centrifugation. Normal bone marrow cells were obtained from healthy donors and used as control cells, through the same procedure.

For the cytokine mRNA production, PBMC were cultured in RPMI 1640 supplemented with 1% FBS, for 24 h without stimulation, and then stimulated with LPS 1ng/ml, IL-1β 10 ng/ml, TNFα 20 ng/ml or poly(I:C) (TLR3 ligand) 25 μg/ml.

For the complementation of patients' fibroblasts with plasmids carrying wild-type MYD88,fibroblasts were incubated with a mixture containing Lipofectamine 2000 reagent (Invitrogen#52887) and plasmids, as previously reported.2 This complex was then removed and fibroblastswere incubated for 18 h in DMEM supplemented with 10% FBS before stimulation. Cytokine Mrnaproduction was assessed in fibroblasts cultured in DMEM supplemented with 10% FBS, incubatedfor 48 h without stimulation, and then with TNF-α, with IL-1β, with poly(I:C) for 6h.

Proliferative assay

Cell proliferation was analyzed by the thymidine incorporation assay. For the evaluation in vitro of proliferative response to mitogens of PMBCs, cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories), phorbol-12- myristate-13-acetate (PMA; 20 ng/ml) ionomycin (0.5 mM) (Sigma Chemical Co) and Interleukin 7 (IL-7; 20/ng/ml).

Cells were plated in triplicate at 1x105 viable cells/well in 96-well plates (BD Biosciences), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine for 8 hours before harvesting and the incorporated radioactivity measured by scintillation counting. Cell viability was determined using trypan blue staining.

Western blot and reagents

Total lysates were obtained from lymphoblastoid B-cell lines generated by EBV immortalization of patients and healthy donors PBMCs. The cells were pre-treated or not with 80nM of Betamethasone for 2h, 0.2 mM H_2O_2 for 30 minutes, or 100nM

Bafilomicyn A1 (Sigma-Aldrich, St. Louis, MO) for 1h, washed with ice-cold PBS (Lonza, Walkersville, MD) and lysed in 100 µl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadatum (Na3VO4), 5 µg ml leupeptin and 5 µg ml aprotinin on ice for 45 min. Fractioned cell lysates were obtained from lymphoblastoid B-cell lines generated by EBV immortalization of patients and healthy donors PBMCs. The cells were treated with 0.2 mM H_2O_2 for 15, 30 or 60 minutes, washed with ice-cold PBS and resuspended in Buffer A (10 mM Hepes/KOH pH 7.9, 1.5 mM $MgCl_2$, 10mMKCl, 0.5mM Dithiothreitol (DTT) pH 7.9, 0.5 mM PMSF, 0.5mM Na3VO4, 5 µg/ml leupeptin and 5 µg/ml aprotinin) on ice for 10 minutes. Cells were centrifuged to collect the supernatant containing the cytosolic fraction. The pellet was resuspended in Buffer C (20 mM Hepes/KOH pH 7.9, 25% glycerol, 420mMNaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM Na3VO4, 5 µg/ml leupeptin and 5 µg/ml aprotinin) on ice for 20 minutes. Cells were centrifuged to collect the supernatant containing the nuclear fraction.

The cell lysates were stored at -80°C. Protein concentration was determined by Bio-Rad Protein Assay, based on Bradford's method. Proteins for LC3, SQSTM1, p-S6K, p-S6 and p-4EBP1 were separated on 4-12% NovexNuPAGE SDS-PAGE gels (Invitrogen, Carlsbad, CA), while proteins for p-mTOR and mTOR were separated on 5% SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes (Sigma-Aldrich, St. Louis, MO). The membranes were then washed three times in wash buffer, blocked and incubated with the specific primary Abs for LC3 (Biorbyt, Cambridge, UK), SQSTM1 (Fitzgerald Industries International, MA, USA), p-S6K (Thr389) (Cell Signaling Technology), p-S6

(Ser235/236) (Cell Signaling Technology MA, USA), p-4EBP1 (Thr37/46) (Cell Signaling Technology, MA, USA), p-mTOR (Ser2448) (Merk Millipore, Germany), m-TOR (Santa Cruz Biotechnology, TX, USA) or Tubulin (Biorbyt, Cambridge, UK). Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked Abs. ECL reagent (Bio-Rad, Woodinville, WA, USA) was used as detection system for visualization. Densitometric analysis was performed using ImageJ software.

Transmission electron microscopy

PBMCs obtained from patients and healthy donors were washed in PBS and centrifuged to obtain a visible pellet. Cells were than fixed with a 1% glutaraldehyde and 0.2M Hepes. After dehydration, from each sample thin sections were cut with a Leica EM UC7 ultramicrotome and further investigated using a FEI Tecnai-12 (FEI, Eindhoven, The Netherlands) electron microscope equipped with a Veletta CCD camera for digital image acquisition at different magnifications.

Autophagosomes were identified on the basis of their ultrastuctural morphology. These vesicles have a double membrane usually visible as two membrane bilayers, which contain cytosol and/or morphologically intact organelles. Autolysosomes were identified on the basis of their characteristic single limiting membrane, containing unwanted cytoplasmic material and/or organelles at various stages of degradation (139).

Fluorescence microscopy

PBMCs obtained from patients and healthy donors were washed with PBS (Lonza, Walkersville, MD) and spotted on sterile coverslips through Shandon CytoSpin III

Cytocentrifuge. Each spot was delimited with the DakoPen (Dako, Denmark). Each section was blocked with normal goat serum before staining and then treated with 1:50 of PerCP-labeled CD3 (BD Pharmingen, San Diego, CA) and 1:100 of FITC-labeled CD45RA Abs (BD Pharmingen, San Diego, CA) or 1:100 LC3 (Biorbyt, Cambridge, UK) and 1:100 LAMP (Biorbyt, Cambridge, UK) antibodies.

Tissue samples were blocked with normal goat serum before staining and then treated with 1:50 of PE-labeled CD4 (Beckman Coulter, Brea, CA) and 1:50 FITC-labeled CD8 (Beckman Coulter, Brea, CA) or 1:50 APC-labeled CD3 (Beckman Coulter, Brea, CA) and FITC-labeled CD7 (Beckman Coulter, Brea, CA),

Appropriate anti-rabbit or anti-mouse peroxidase-linked secondary Abs were used. Nuclear counterstain was visualized with DAPI (4',6-diamidino-2-phenylindole, 0.05 mg/ml, (Vector Laboratories, CA, USA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

Real-time quantitative reverse transcriptase PCR analysis

Total RNA from patients and controls PBMCs, from normal human skin fibroblasts, normal human thymus, CB mononuclear cells (CBMCs) or intestinal frozen tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and Phase-lock gel columns (Eppendorf, Germany) according to the manufacturer's instructions. RNA was reverse transcribed by Transcriptor First Strand Cdna Synthesis Kit (Roche, Germany). The Qpcr reactions were performed in duplicate. The amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche Applied Systems, Germany). Cycling conditions comprised an initial denaturation at 94°C for 5 min, a phase of annealing/extension specific for each gene. A dissociation procedure was performed to generate a melting curve for

confirmation of amplification specificity. Primers used are listed in Table 6. Results are mean ± standard error (SE) of 2 repeat experiments and each gene expression was normalized to β-actin as housekeeping gene. The relative transcript abundance was represented as –DCt = (Ct gene – Ct reference) and the relative changes in gene expression was analyzed using the 2^{-DDCt} method (140).

Table 6. Sequences of oligos used in Real-Time qPCR analysis.

	Primers Sequence (5'-3')	
GENE	**Forward primer**	**Reverse primer**
UVRAG	TGACAATTCGTTGCAGGCAGTTA	AGGCAACTTGACACCGCATACA
VPS11	CCACTTTGATGTGGAGACAGC	TGTATCGAAGGGCTTCCTGA
VPS 18	AGCGTCGCTACCTGGAGAG	GTACGTTCGGCTGGCTTC
CSTF	ACAGAGGAGGAGTTCCGCACTA	GCTTGCTTCATCTTGTTGCCA
CSTB	AGTGGAGAATGGCACACCCTA	AAGAAGCCATTGTCACCCCA
CSTD	AACTGCTGGACATCGCTTGCT	CATTCTTCACGTAGGTGCTGGA
CSTA	CAGGCTTTGGTCTTCTCTCCA	TCACGCATTCCAGGTCTTTG
CSTE	GGACATGATCCAGTTCACCGA	GGTTGGCACACTCCACAGCA
GBA	TGGGTACCCGGATGATGTTA	AGATGCTGCTGCTCTCAACA
GUS	ATTGGAGGTGCAGCTGACTG	TCCTCATGCTTGTTGACACC
CD63	TCACGTTTGCCATCTTTCTG	TCGAAGCAGTGTGGTTGTTT
KIF1Bβ	GACCAAGCTCAGTGGTCTCTAAG	CCAGATGACCTGGTCGTATTGTGC
KIF2A	GCCTTTGATGACTCAGCTCC	TTCCTGAAAAGTCACCACCC
ARL8A	AGTCCTGGGTAACAAGCGAGAC	GCAAGAGATGGAGTAGCAGCAG
ARL8B	CACCTTCGTCAATGTCATCG	CCTATGTCCCAGATCTTTATTGTG
KIF3C	GAAGATGCTGGAGGACCTGCGG	GTAGGTGCCCCGGAGCTCCATAG
IL7Rα	GGAGCCAATGACTTTGTGGT	CTGCAGGAGTGTCAGCTTTG
IL2RG	TGCTAAAACTGCAGAATCTGGT	AGCTGGATTCACTCAGGTTTG
CyclinD1	AGGTCTGCGAGGAACAGAAGTG	TGCAGGCGGCTCTTTTTC

CyclinD2	CTGTGTGCCACCGACTTTAAGTT	GATGGCTGCTCCCACACTTC
CyclinD3	GCAGCGCCTTTCCCAACT	TCAAAAGGAATGCTGGTGTATGTATC
BCL-XL	GTAAACTGGGGTCGCATTGT	TGCTGCATTGTTCCCATAGA
Beclin-1	GGCTGAGAGACTGGATCAGG	CTGCGTCTGGGCATAACG
CD3ε	GATGCAGTCGGGCACTCACT	TTGGGGGCAAGATGGTAATG
RAG1	CATCAAGCCAACCTTCGACAT	CAGGACCATGGACTGGATATCTC
RAG2	CCTGAAGCCAGATATGGTC	GTGCAATTCACAGCTGGGCT
pTα	CATCCTGGGAGCCTTTGGT	CCGGTGTCCCCCTGAGAG
β-actin	GACAGGATGCAGAAGGAGAT	TTGCTGATCCACATCTGCTG

Flow cytometry analysis

In flow cytometry of PBMCs, patients and healthy donors cells were exposed to directly-conjugated mouse anti-human monoclonal antibodies (mAbs) with CD45-APC-Cy7 (BD Biosciences), CD3-PeCy5 (BD Biosciences), and CD-127-AlexaFluor 488 (Biolegend). The cells were incubated with directly-labelled antibody clones at 4°C in the dark for 30 min, washed and resuspended in 100 ml PBS. The events in the displayed graphs and dot plots were gated by forward and side scatter to exclude dead cells. Analytical flow cytometry was performed using a BD FACS Canto II flow cytometer (BD Biosciences). Subsequent data processing and preparation for presentation was done using BD FACSDiva software.

In flow cytometry of CBMCs, FITC-, phycoerythrin (PE)-, allophyocyanincyanine 7 (APC-Cy7)-, peridin chlorophyll protein (PerCP)- or PECy7-coupled Abs were used on CB toward CD45 (2D1), CD7 (MT701), CD2 (RPA-2.10), CD3 (UCHT1), CD8a (SK-1), CD8b (2ST8.5H7), CD4 (L200), CD62L (SK11), CD45RA (HI100), CD27 (L128), CD45RO (UCHL-1), CD103 (Ber-ACT8) from BD Pharmingen, San Diego, CA or Beckman Coulter, Brea, CA. FACSCanto II flow cytometer and FACSDiva

software (BD Bioscience, San Jose, CA) were used. For each sample, negative controls were stained with irrelevant Abs conjugated with the same fluorochrome. The ‘‘fluorescenceminus-one’’ (FMO) controls have also been used to define precisely the cells that have fluorescence above background levels. Briefly, the samples have been stained with all of the reagents except one.

DNA extraction

Total DNA was isolated from peripheral blood lymphocytes with DNA Blood Mini Kit_ (Qiagen, Hilden, Germany). Quantity and quality were determined on the Nano-Drop_ ND-1000 spectrophotometer (ThermoScientific,Waltham, MA, USA).

Targeted Next Generation Sequencing

Targeted next generation panel, "TaGSCANv.2" (Targeted Gene Sequencing and CustomAnalysis) was run, which includes 572 genes. Briefly, samples were prepared for sequencing usingTruSight Rapid Capture with TruSight Inherited Disease Oligos (Illumina); the MT genome iscovered at 1000x by the addition of MT oligos during enrichment (IDT). Samples were sequencedto at least 2.5Gb on an Illumina MiSeq with TruSeq v3 reagents, yielding paired 250 nucleotidereads. Alignment, variant calling, and analysis were performed as previously described (141). In this case, analysis was customized for immunodeficiency, which limited the genes examined to 55 (Table 7). Variant analysis was confined to coding and splice variants with a minor allele frequency (MAF) of 1% or less in the CMH internal database. Sanger sequencing was used to verify the finding and confirm carrier status of both parents.

Table 7. List of the 55 studied genes in Targeted Next Generation Sequencing

ADA
AP3B1
ATM
BLM
BLOC1S6
BTK
CD19
CD247
CD3D
CD3E
CD3G
CD40LG
CFP
DCLRE1C
DOCK8
ERCC2
ERCC3
FERMT3
FOXN1
FOXP3
G6PC3
GTF2H5
ICOS
IFNGR1
IFNGR2
IKBKG
IL12B
IL12RB1
IL1RN
IL2RG
JAK3
LIG4
LYST
MPV17
MYD88
NHEJ1
ORAI1
PRF1
RAB27A
RAG1
RAG2
RMRP
SH2D1A
SLC35C1
SP110
STAT1
STIM1
STX11
ADA
AP3B1
ATM
BLM
BLOC1S6
BTK
CD19

Statistical analysis

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

SUMMARY

The immune system is a complex integrated network of chemical and cellular mediators that developed during evolution to defend the body from any form of chemical, traumatic or infective insult to their integrity.

A proper immune response relies on the innate immunity, that is responsible for a first line of defense against aggression and the aspecific recognition of a limited repertoire of antigens, and, later, on the adaptative immunity which includes chemical and cellular mediators responsible for a more powerful and specific defensive response from any form of antigen. Alterations of any part of the immune response results in failure of host defense and, in particular, of immunodeficiency, autoimmunity and cancer predisposition.

Primary immunodeficiency disorders (PIDs) are rare inherited disorders characterized by poor or absent function in one or more components of the immune system, that result in chronic, recurrent and life-threatning infections if not promptly diagnosed and treated (2). Traditionally, PIDs are classified according to the component of the immune system that is primarily disrupted: innate or adaptive immunity. In the last 20 years, thanks to the progress in molecular technologies, a remarkable improvement of the knowledge in the field of PIDs, concerning both their pathogenetic mechanisms and clinical features, has been observed. Nowadays, about 300 forms of well-characterized PIDs have been identified underlying complex phenotypes which encompass a wide spectrum of clinical features ranging from recurrent bacterial infections to other unusual manifestations, such as autoimmune disorders, cancer susceptibility, allergy and auto inflammation (8, 142). However, a recent study reported that PIDs are underdiagnosed and, therefore, they are

presumably more common than previously estimated. Thus far, the diagnosis of a specific PID has been based onthe demonstration of a functional immune defect and on the subsequent identification of candidate genes, which are selectively involved in the biochemical pathway implicated in that specific functional alteration.

In this thesis, during the three years of my PhD program, I have contributed to elucidate "*Congenital Immunodeficiencies: novel diagnostic and therapeutic approach*", through the clinical, cellular, functional and molecular study of some Immunological disorders. In particular, my research work has been focused on the study of the potential involvement of autophagy in the pathogenesis of Ataxia Telangiectasia, and the potential positive effect of glucocorticoids on the rescue of a proper cell clearance process in lymphocytes of the patients affected with this disease.

Moreover, I contributed in the characterization of the potential oncogenic role of the common gamma chain (γc) in primary human hematopoietic malignant cell lines, by evaluating the molecular mechanisms by which this protein promotes tumor growth.

In addition I contributed to better define the role of the epithelial cells in the immune system and of thymic stromal alterations responsible for immunological disorders. Eventually I participated to two studies focused on the application of Next Generation Sequencing technique for the diagnosis of immunodeficiencies.

Overall, the results obtained during my PhD course could be useful both in the clinical practice and in the basic research of immunological diseases.

europass Curriculum vitae

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EDUCATION AND TRAINING

02/2013–02/2016

PhD

ISCED7

Unit of Immunology, Department of Translational Medical Sciences at the "Federico II" University of Naples, via Pansini, 5 – 80131, Naples (Italy)

PhD student at the Doctoral Course in "Human Reproduction, Development and Growth" with a research project focused on the evaluation of the role of autophagy in the pathogenesis of Ataxia Teleangiectasia.

12/2008–03/2011

Bachelor in Biological Sciences (II level degree) at the "Federico II" University of Naples, with a thesis entitled: "Valutazione funzionale di mutazioni del gene della Glucochinasi (GSK): approccio metodologico". Vote: 110/110 with honors.

"Federico II" University of Naples, via Pansini, 5 – 80131, Naples (Italy)

Internship at Department of Biochemistry and Medical Biotechnology of the "Federico II" University of Naples, Naples, Italy, focused on the functional characterization of two mutations of the GCK gene, identified in patients affected with Maturity Onset Diabetes of the Young (MODY2).

10/2005–12/2008

Bachelor in General and Applied Biology, (I level degree) at the "Federico II" University of Naples with a thesis entitled: "Isolamento di sottopopolazioni di cellule dendritiche mediante cell-sorting". Vote: 104/110

ISCED6

"Federico II" University of Naples, via Cinthia, 21 – 80126, Naples (Italy)

Internship at the Department of Biology of the "Federico II" University of Naples, Naples, Italy, focused on the isolation of dendritic cells from Peripheral Blood Mononuclear Cells (PBMCs) through cell-sorting.

07/2007–06/2008

Winner of the "Competition for the assignment of part-time collaboration- Academic Year 2007/2008- with the duty to commit 150 hours of activity at the University Library of Sciences of the "Federico II" University of Naples, via Cinthia – 80131, Naples – Italy.

"Federico II" University of Naples, via Cinthia – 80131, Naples (Italy)

The main activities were:

- management of the access of students to the study area of University Library of Sciences.
- management of the request of books/collections present in the Library.

PERSONAL SKILLS

Mother tongue(s) Italiano

Other language(s)

UNDERSTANDING		SPEAKING		WRITING
Listening	Reading	Spoken interaction	Spoken production	
Inglese	C1	C1	B2	B1	C1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills

Predisposition to work in a team of 5-6 persons by collaborating each other in a friendly manner and capacity to interact with other colleagues also in multicultural environments, developed during my PhD studies.

Organisational / managerial skills

Capacity to design a scientific project including the economic budget. (See the attached list of application to grant proposals) Capacity to administrate small budgets for the daily work in a small lab. Capacity to coordinate students in their practice in laboratory also by follow them in the preparation of the thesis. Capacity to contribute to the coordination and management of the scientific revision of articles required by international scientific journals, as "Frontiers in Immunology" e "International Reviews of Immunology".

Job-related skills

- Cell cultures
- PBMC purification by Ficoll
- Primary and continue human cell lines manipulation
- Primary culture of skin derived fibroblast preparation
- B lymphocyte immortalization with EBV infection
- Cell proliferation assay (thymidine)
- Death assay (Trypan blue)
- Western blot
- Immunofluorescence
- Basal knowledge of multiparametric flow cytometry analysis
- DNA and RNA extractions (cells and tissues)
- PCR, Agarose gel electrophoresis, Sequencing analysis
- Reverse transcriptase and Real_time PCR
- Transfection and RNA interference with lipofectamine

Other skills

Knowledge of entrezgene, genecards, embl nucleotide sequence database and of the UCSC genome browser.
Ability to perform a proliferative assay through the evaluation of thymidine incorporation by lymphocytes pre-stimulated with mitogens for diagnosis of the immunodeficiencies.
These competences were acquired during my training at the Department of Translational Medical Sciences, where I also participated to clinical practice by diagnosing some immune disorders.
Use of endnote 7.0 to format and add references to a manuscript. Use of Word, graphical softwares such as PowerPoint, Publisher,Photoshop, statitistical softwares such as Excel e GraphPad Prism and softwares to elaborate images such as ImageJ.I also participated to the preparation of an entire scientific paper also by creating imagines and graphics and to the preparation of lessons, seminars and posters for congress and meetings (see the attached list of scientific production). Use of databases of scientific articles, in particular SCOPUS and ISI Web of Science and ability to perform the h-index and contemporary index calculi for the evaluation of scientific production. Use of the University program "IRIS" for the management of scientific publications of professors.These competences were acquired during the PhD at the Department of Translational Medical Sciences, where I also contributed to the "Evaluation of the Quality of the Research" 2011-2014 (VQR 2011-2014).

ADDITIONAL INFORMATION

Scientific Interests

- Primary Immunodeficiencies: definition of novel therapeutical strategies for the treatment of Ataxia Teleangiectasia.
- Regulatory mechanisms governing lymphocyte cell proliferation, activation and cell death. In particular, the study role of γc in cell cycle progression, strongly related to its cellular amount;
- Novel aspects in immunodeficiencies, with a particular regard to Severe Combined immunodeficiency (SCID) and molecular analysis of genes whose mutations are responsible for certain immunodeficiencies;
- Studies of previously unappreciated relationships between receptor signaling systems in the pathogenesis of SCIDs and signal transduction in physiology and human diseases affecting the immune system.

SCIENTIFIC SOCIETIES

European Society for Immunodeficiencies (ESID) Junior Member

APPLICATIONS TO GRANT PROPOSALS

1. Futuro in Ricerca-Call for MIUR Application 2013 with a project entitled: "Characterization of the potential oncogenic role of γc by exploring its modulatory activity on genes related to growth signaling, cell cycle control and survival, and through its post-transcriptional miRNAs-mediated regulation".
2. Sparks Project grant application for A-T 2013 with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its pathogenic implication in Ataxia-Telangiectasia: potential modulatory effect of Betamethasone".
3. Telethon Grant Proposals-Call for Applications 2014 with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its pathogenic implication in Ataxia-Telangiectasia: potential modulatory effect of Betamethasone".
4. Telethon Grant Proposals-Call for Applications 2015 with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its role in the pathogenesis of Ataxia Telangiectasia: potential modulatory effect of Betamethasone or other FDA approved drugs evaluated through High Content Screening".
5. PRIN - Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2015, with a project entitled: "Targeted next generation sequencing as an approach to patients with severe forms of congenital immunodeficiencies".
6. Jeffrey Modell Foundation – Grant for Immunodeficiency Disorders on Trialect, with a project entitled: "*In vitro* development of medullary thymic

epithelial cells (mTECs) obtained by the Reprogramming technology, to support HSC differentiation into fully mature single positive T cells on a 3D thymic organoid".

7. Action for A-T - Call for Ataxia Telangiectasia research applications with a project entitled: "Evaluation of the link between autophagy and Ataxia-Telagiectasia and of potential modulatory effect of Betamethasone or other FDA approved drugs evaluated through High Throughput Screening".

SCIENTIFIC PRODUCTION

LIST OF PUBLICATIONS:

1. De Martino L., Capalbo D., Improda N., D' Elia F., Di Mase R., **D'Assante R.**, D' Acunzo I., Pignata C., Salerno M. APECED: A paradigm of complex interactions between genetic background and susceptibility factors. Front Immunol. 4:331, 2013.
2. Fusco A., Panico L., Gorrese M., Bianchino G., Barone M.V., Grieco V., Vitiello L., **D'Assante R.**, Romano R., Palamaro L., Scalia G., Del Vecchio L., Pignata C. Molecular evidence for a thymus-independent partial T cell development in a FOXN1-/- athymic human fetus. PLoS One 8(12):e81786, 2013.
3. Pignata C., **D'Assante R.**, Sousa AE. Thymic stromal alterations and genetic disorders of immune system. Front Immunol. 6:81, 2015.
4. **D'Assante R.**, Fusco A., Palamaro L., Giardino G., Gallo V., Cirillo E., Pignata C. Unraveling the link between ectodermal disorders and primary immunodeficiencies. Int Rev Immunol. [Epub ahead of print], 2015.
5. Cirillo E., Giardino G., Gallo V., **D'Assante R.**, Grasso F., Romano R., Di Lillo C., Galasso G., Pignata C. Severe combined immunodeficiency-an update. Ann N Y Acad Sci. 1356(1):90-106, 2015.
6. Giardino G., Gallo V., Somma D., Farrow E.G., Thiffault I., **D'Assante R.,** Donofrio V., Paciolla M., Ursini M.V., Leonardi A., Saunders C.J., Pignata C. Targeted next generation sequencing revealed MYD88 deficiency in a child with chronic Yersiniosis and granulomatous lymphadenitis. J Allergy Clin Immunol. IN PRESS, 2015.

MEETING ABSTRACTS AND COMMUNICATIONS:

1. Cirillo E., Gallo V., Giardino G., Galasso G., Romano R., **D'Assante R.**, Genesio R., Baldini A., Nitsch L., Pignata C. DiGeorge-like syndrome in a child with a 3p12.3 deletion involving miRNA-4273 born to a diabetic mother. XVI meeting of the European Society for Immunodeficiencies, Prague 29 October-1 November 2014. (Poster)
2. Giardino G., Cirillo E., Gallo V., **D'Assante R.**, Paciolla M., Ruggiero G., Ursini M.V., Carsetti R., Puel A., Pignata C. Chronic mucocutaneous candidiasis, recurrent herpetic infections and suppurative eyelid infections in a patient carrying a novel gain-of-function mutation in the STAT1 DNA-binding domain. XVI meeting of the European Society for Immunodeficiencies, Prague 29 October-1 November 2014. (Poster)
3. Gallo V., Cirillo E., Giardino G., **D'Assante R.**, Spennato P., Cinalli G., Pignata C. Intrathecal amphotericin B therapy in a patient with X-linked chronic granulomatous disease and refractory cerebral invasive aspergillosis. XVI meeting of the European Society for Immunodeficiencies, Prague 29 October-1 November 2014. (Poster)
4. Romano R., **D'Assante R.**, Bianchino G., Grieco V., Parasole R., Poggi V., Palamaro L., Fusco A., Scotto Di Marco G., Pignata C. Gamma chain is prominently overexpressed in B-pre acute lymphoblastic leukemia cells. XVI meeting of the European Society for Immunodeficiencies, Prague 29 October-1 November 2014. (Poster)
5. Fusco A., Polishchuk E., **D'Assante R.**, Palamaro L., Ballabio A., Pignata C. Aberrant autophagic vesicles in the lymphocytes from patients affected with

Ataxia-Telangiectasia. XVI meeting of the European Society for Immunodeficiencies, Prague 29 October -1 November 2014. (Poster)

6. **D'Assante R.**, Fusco A., Polishchuk E., Palamaro L., Ballabio A., Pignata C. Accumulation of autophagic vesicles in lymphocytes from patients affected with A-T. Ataxia Telangiectasia Clinical Research Conference 2014, Nijmegen, the Netherlands, 13-15 November 2014. (Oral presentation)
7. Fusco A., **D'Assante R.**, Scalia G., Palamaro L., Del Vecchio L, Pignata C. The low proliferation rate of lymphocytes from A-T patients directly correlates to the reduced surface expression of IL7Rα trafficked through autophagic vescicles. Ataxia Telangiectasia Clinical Research Conference 2014, Nijmegen, the Netherlands, 13-15 November 2014. (Poster)

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2. Notarangelo LD, Fischer A, Geha RS, Casanova JL, Chapel H, Conley ME, et al. Primary immunodeficiencies: 2009 update. J Allergy Clin Immunol. 2009;124:1161-78.

3. Palmer K, Green TD, Roberts JL, Sajaroff E, Cooney M, Parrott R, et al. Unusual clinical and immunologic manifestations of transplacentally acquired maternal T cells in severe combined immunodeficiency. J Allergy Clin Immunol. 2007;120:423-8.

4. Zannolli R, Sabrina B, Betti G, Salvucci S, Plebani A, Soresina A, et al. A randomized trial of oral betamethasone to reduce ataxia symptoms in ataxia telangiectasia. Mov Disord. 2012;27:1312-6.

5. Broccoletti T, Del Giudice E, Amorosi S, Russo I, Di Bonito M, Imperati A, et al. Steroid-induced improvement of neurological signs in ataxia-telangiectasia patients. Eur J Neurol. 2008;15:223-8.

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7. Russo I, Cosentino C, Del Giudice E, Broccoletti T, Amorosi S, Cirillo E, et al. In ataxia-teleangiectasia betamethasone response is inversely correlated to cerebellar atrophy and directly to antioxidative capacity. Eur J Neurol. 2009;16:755-9.

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