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PH.D. PROGRAM IN

CLINICAL AND EXPERIMENTAL MEDICINE

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(Years 2015-2018)

Chairman: Prof. Francesco Beguinot

Ph.D. Thesis

"Novel insights in the pathogenesis of congenital immunodeficiencies"

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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 an increased susceptibility to cancer. In particular, a high cancer susceptibility has been reported for a rare form of PID called Ataxia Telangiectasia (A-T) whose clinical hallmark is represented by the cerebellar neurodegeneration with the loss of Purkinje cells. Recently, in a few clinical trial sit 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 both T 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 "Clinical and Experimental Medicine" (XXXI Cycle, years 2015-2018). During these years my research has been focused on the study of the following lines of research:

- positive effect of oral betamethasone administration on the *in vitro* lymphocytes functionality in patients affected with Ataxia-Telangiectasia, and the identification of the molecular checkpoint responsible for the partial functional rescue in lymphocytes of the patients affected with this disease.
- characterization of a novel immunodeficiency whose hallmarks are represented by high IgM levels, impaired B-cell homeostasis and cancer susceptibility,
- autoimmune manifestations and the pathogenetic mechanism underlying autoimmunity in a specific PID.

CHAPTER I

“In Ataxia-Telangiectasia, the functionality of the IL-7/IL-7Rα axis parallels the neurological behavior during in vivo Betamethasone treatment”

Introduction

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 repair could 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).

A short-term treatment with glucocorticoids is able to transiently rescue the A-T neurological phenotype and to increase lymphocytes proliferation, even though the mechanism of action has not yet been fully defined (5, 6, 7). However, these effects have been observed only in a few patients, the neurological improvement being inversely correlated with the extent of cerebellar atrophy and positively correlated with the antioxidative capacity (7). It should be noticed that the variability was not explained by the amount of ATM protein, which was absent in both the responders and non-responders. The effect on cell proliferation represents a paradox, since it is in contrast with the well-known immunosuppressive role of glucocorticoids.

IL-7/IL-7R signaling pathway represents a central checkpoint in the cell cycle progression of lymphocytes. IL-7R expression is dynamically controlled in T- and B-cell development and is highly expressed on naive and memory CD4+ and CD8+ T cells (20, 21). When naive T cells

receive Ag stimuli, IL-7Rα is down-regulated and the cells become effector T cells (22). The IL-7R-chain (IL-7R, CD127) is a prominent gene target of glucocorticoids in that it is up-regulated in response to glucocorticoids (23), while it is rapidly down-regulated in response to a number of stimuli, including IL-7 (24). A decreased IL-7R expression on activated effector T cells ensures that they are short lived and prone to apoptosis, while high expression of IL-7R on naive and memory T cells ensures their survival over the long term (25, 26).

IL-7R, as other signal-transducing cell-surface receptors, are constitutively recycled at the cell membrane and, following their binding to the ligand, are internalized through an endocytosis mechanism (27). Thereafter, these proteins are sorted in early endosomes and are either returned to the cell surface or degraded by proteasomes and/or lysosomes (28).

Aims

This study was aimed to verify whether the positive influence of *in-vivo* administered low doses of GC on the lymphocyte proliferative response correlated with the neurological positive response and whether the IL-7/IL-7R axis signaling pathway is implicated in the process.

Materials and methods

Patients

Eleven A-T patients were initially studied for the proliferative response to PHA or IL-7. Four of these 11 patients, referred at a single Center, were enrolled in a multicenter clinical trial, aimed at defining the minimal effective dosage of oral Betamethasone at the dosage of 0.001, 0.005 or 0.01 mg/kg/day (29). Neurological assessment was performed through the Scale for the Assessment and Rating of Ataxia (SARA) at each time-point. In the already published study, all patients were categorized as responders, partial responders and non-responders. In this study, patients of a single center were further studied at immunological level. PBMCs were collected at specific time points during the treatment period.

Cell Culture

PBMCs were obtained from 11 A-T patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. PBMCs 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 μg/ml gentamycin (Gibco, Carlsbad, California), 10% Penicillin-Streptomycin (Lonza, Verviers, Belgium), and cultured at 37°C, 5% CO2. PBMCs were put in culture (2 * 10^5 / well) in triplicate in 96-well plates in RPMI 1640 containing 10% FBS, 2mM/L L-glutamine and 50 μg/ml gentamycin, with or without PHA (8

μg/ml) and IL7 (20 ng/ml), for 72 hours. All the experiments were approved by the Ethical Committee for Biomedical Activities of Federico II University, trial registration n. 55/14; Eudract n. 2014-000454-10.

Flow cytometry

PBMCs were collected according to informed consent procedure. Lymphocytes were gated on a (CD45) forward scatter vs. side scatter dot plot. CD127+, CD25+, CD69+ and HLA-DR+ cells were gated from the CD3+ lymphocyte population. Cells were acquired by a flow cytometer FACS-Canto II (Becton Dickinson, San Jose, CA, USA), equipped with three lasers and eight photomultipliers and analyzed with the BD FACSDiva software.

Real-time quantitative reverse transcriptase PCR analysis

Total RNA from A-T 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 the Table 1. Results are expressed as mean $\pm$ standard error (SE) 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 (30).

Western blotting

Total lysates were obtained from PBMCs of A-T patients at all time points during the clinical trial. The cells were washed with ice-cold PBS (Lonza, Walkersville, MD) and lysed in 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. Protein concentration was determined by Bio-Rad Protein Assay, based on Bradford's method. Proteins were separated on 4-12% NovexNuPAGE SDS-PAGE gels (Invitrogen, Carlsbad, CA. Proteins were 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 antibodies for IL-7Rα (R&D System, MN, USA) or Actin (Sigma-Aldrich, St. Louis, MO). Immune complexes were detected using the appropriate anti-mouse peroxidase-linked antibodies. ECL reagent (Bio-Rad, Woodinville, WA, USA) was used as detection system for visualization.

Confocal microscopy

PBMCs obtained from A-T patients 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 a mix of 1:25 IL-7Rα (R&D System, Minneapolis, USA) and 1:300 EEA1 (Santa Cruz Biotechnology, TX, USA) or Rab-7 (Santa Cruz Biotechnology, TX, USA) rabbit antibodies. Appropriate anti-mouse or anti-rabbit peroxidase-linked secondary antibodies were used. Nuclear counterstain was performed with 0.05 mg/ml DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, CA, USA). Images were acquired by a confocal microscope (LSM 510, Zeiss, Germany).

Statistics

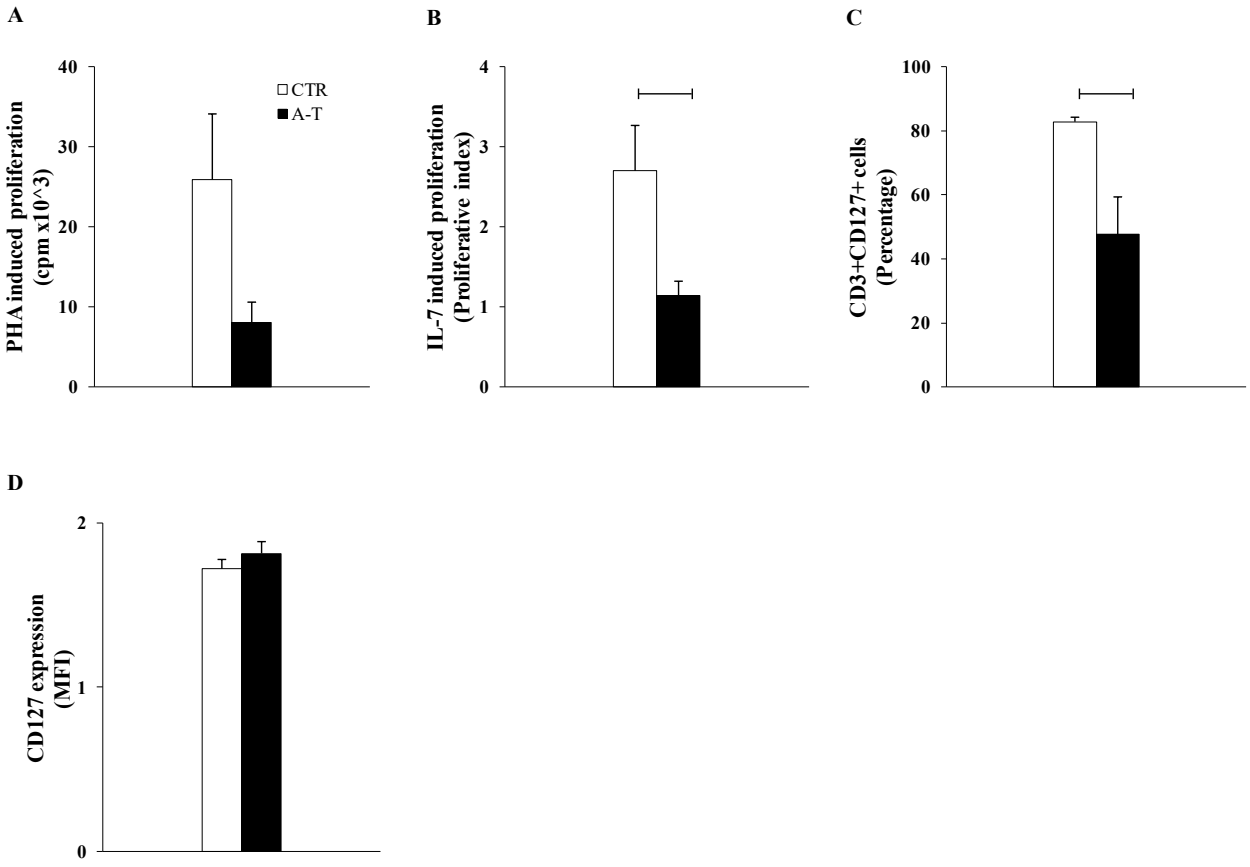
GraphPad Prism software was used for data analysis. The Student's *t*-test was used to analyze the statistical significance of differences. The minimum acceptable level of significance was $p \leq 0.05$ calculated through two-tailed unpaired Student's *t*-test.

RESULTS

Evaluation of the proliferative response in lymphocytes from patients affected with A-T

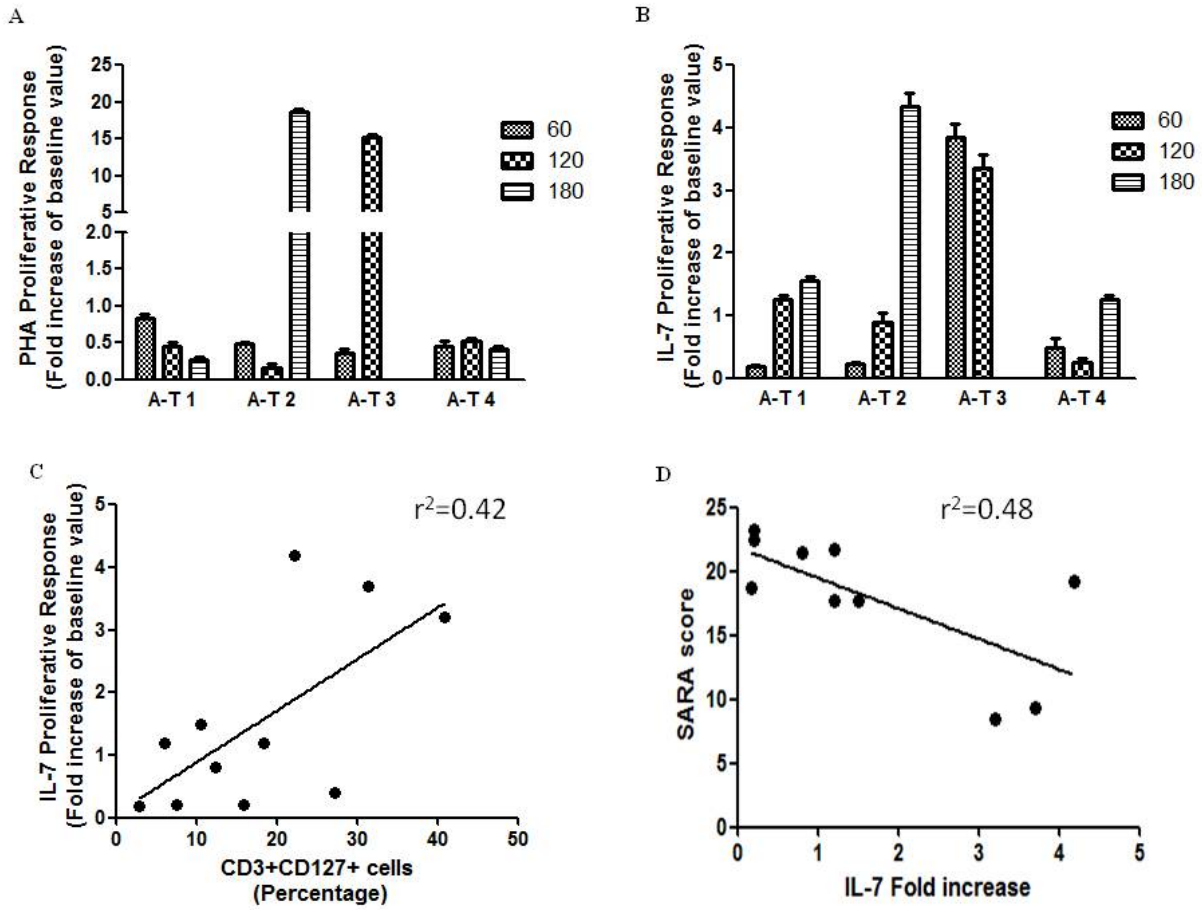
Immune deficiency in A-T patients is characterized by poor lymphoproliferative ability. In our cohort of A-T patients before the GC therapy (n = 11), the proliferative response to the mitogen PHA was reduced by 69% as compared to healthy controls (cpm mean value ± SD: 8061 ± 2549 *vs* 25907 ± 8192) (Figure 1A). Similarly, the proliferation following Il-7 stimulation was lower in A-T patients than in controls (n = 13) (Figure 1B). The proliferative index (PI) in patients and controls was 1.14 ± 0.18 (mean ± SD) and 2.70 ± 0.56, respectively (p = 0.028). We next evaluated whether the reduced proliferation to IL-7 correlated to the percentage of circulating CD3+ cells expressing IL-7Rα (also known as CD127). In A-T patients, 47.7% of $CD3^+$ gated cells expressed CD127, while in the controls they were 82.2% (p = 0.017) (Figure 1C). Moreover, the mean fluorescence intensity of $CD127^+$ cells was comparable in both groups (mean ± SD: 1.7 ± 0.09 *vs* 1.7 ± 0.06) (Figure 1D).

FIGURE 1


A
PHA induced proliferation (cpm x10^3)
40
30
20
10
0
CTR
A-T
B
IL-7 induced proliferation (Proliferative index)
4
3
2
1
0
C
CD3+CD127+ cells (Percentage)
100
80
60
40
20
0
D
CD127 expression (MFI)
2
1
0

The immune deficiency is variable among A-T individuals (Chopra et al., CEI 2014) and we confirmed that, although the poor lymphoproliferative ability was constant in the same subject over the time (data not shown), it was highly variable among different patients. Since a similar variability was also observed in the neurological response to oral betamethasone administration, we wondered whether there was a correlation between the behavior of lymphoproliferative ability and the neurological response to the drug. Thus, by taking advantage of a multicentric clinical trial aimed at defining the minimal effective dosage of oral steroid {Cirillo, 2018 #3819}, we compared in the 4 A-T patients, referred at a single Center, the immunological and neurological behavior during the betamethasone trial. The evaluation of the proliferative response to PHA showed a 19- and 15-fold increase only in 2 of the 4 A-T patients (Figure 2A). Similarly, the proliferative response to IL-7 showed a 3- to 4-fold increase in the same patients, at the different time points, and again no increase was noted in the other patients (Figure 2B). A correlation between the proliferative response to IL-7 and the percentage of CD3+CD127+ cells was found (Figure 2C). Of note, as shown in the Figure 2D, a significant inverse relationship between the proliferative response to IL-7 and the behavior of the SARA score was found.

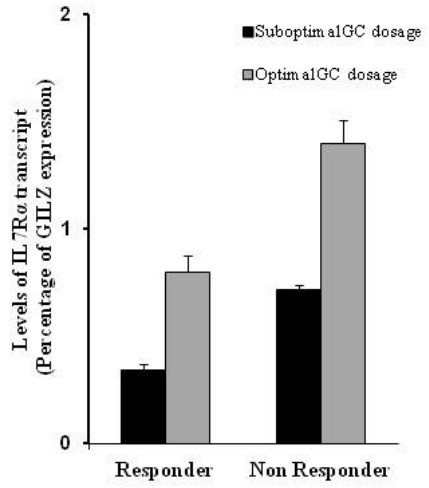
FIGURE 2


A
PHA Proliferative Response
(Fold increase of baseline value)
25
20
15
10
5
2.0
1.5
1.0
0.5
0.0
A-T 1
A-T 2
A-T 3
A-T 4
60
120
180
B
IL-7 Proliferative Response
(Fold increase of baseline value)
5
4
3
2
1
0
A-T 1
A-T 2
A-T 3
A-T 4
60
120
180
C
IL-7 Proliferative Response
(Fold increase of baseline value)
r2=0.42
0
10
20
30
40
50
CD3+CD127+ cells
(Percentage)
D
SARA score
r2=0.48
25
20
15
10
5
0
1
2
3
4
5
IL-7 Fold increase

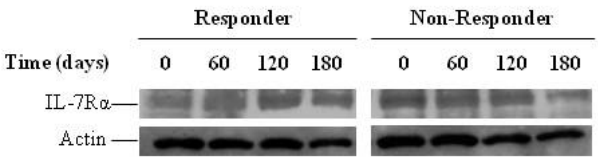
Since the expression of IL-7Rα varied among A-T patients, we evaluated through Real-Time PCR the expression levels of *IL-7Rα* mRNA in the most responder and the non-responder at all to GC. At the transcription level, no difference was found in the IL-7Rα mRNA, expressed as percentage of the GILZ steroid target, between the patients (Figure 3A). Similarly, as shown in the Figure 3B the amount of the protein in the cytoplasm was comparable between the patients, thus supporting what observed at the transcription level.

FIGURE 3

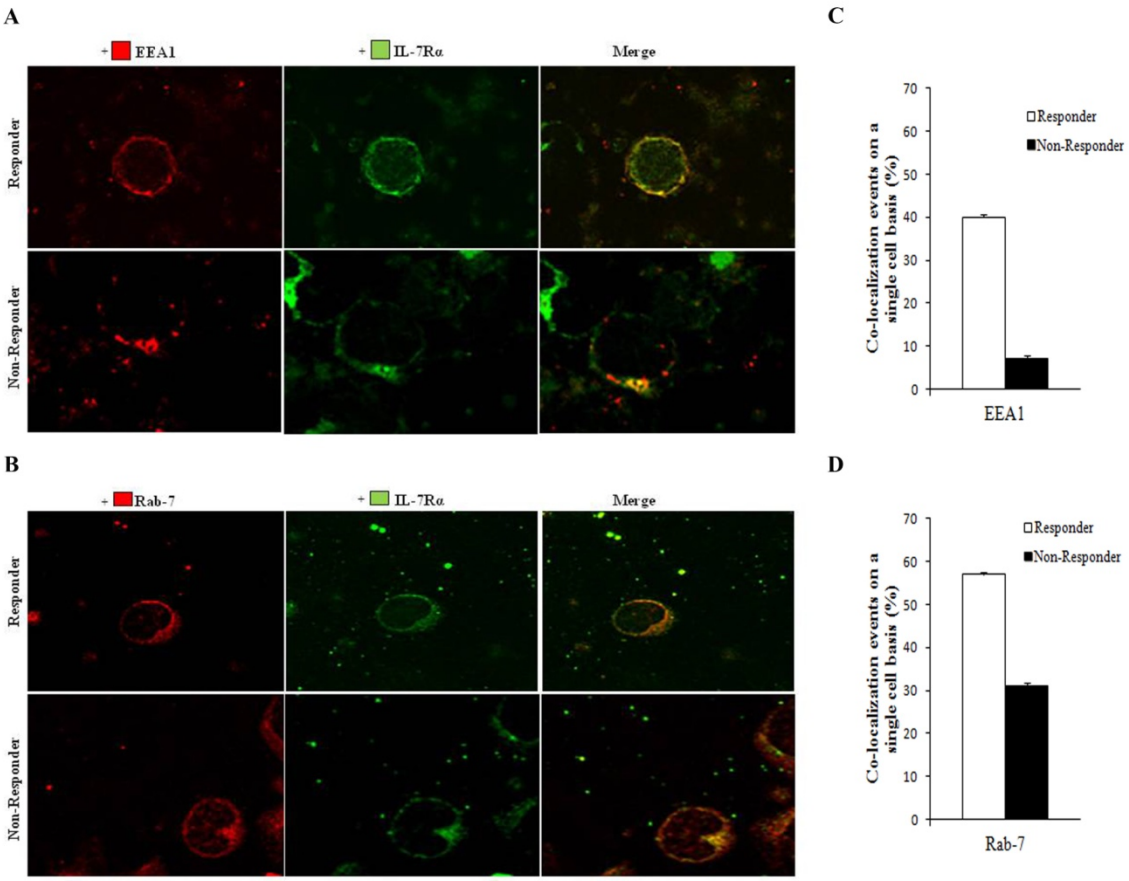
A


Suboptimal GC dosage
Optimal GC dosage
Levels of IL7Rα transcript
(Percentage of GILZ expression)
2
1
0
Responder
Non Responder

B

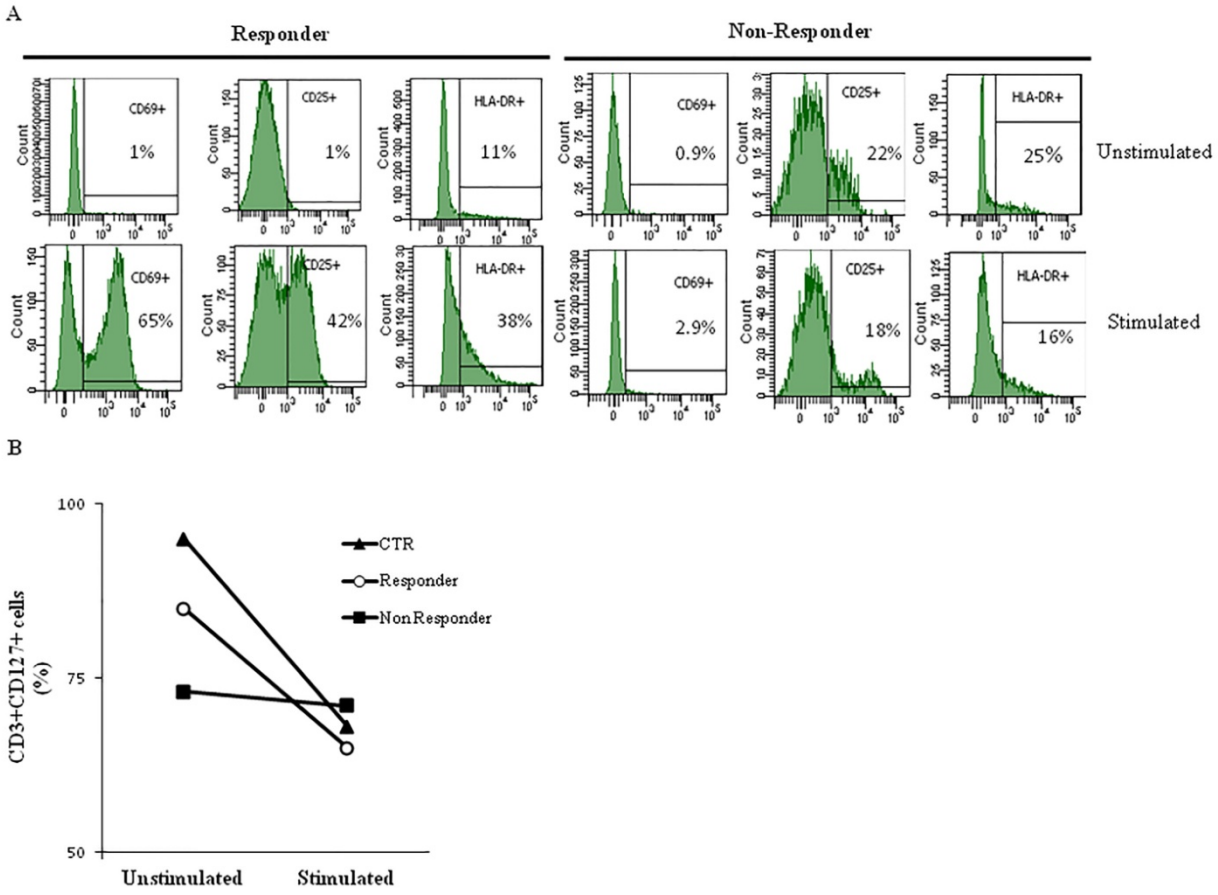

Responder
Non-Responder
Time (days)
0
60
120
180
0
60
120
180
IL-7Rα
Actin

We next analyzed through confocal microscopy the subcellular localization of the molecule in the most responder and the non-responder at all to GC. We found that IL-7Rα was expressed in responder and non-responder patients in a similar fashion (Figure 4A,B). However, in the responders most of the molecule (40%) was localized into the early endosome (EEA1) vesicles, differently from the non-responders, in whom the co-localization events on a single cell basis was only 7% (Figure 4C). We next evaluated the amount of IL-7Rα internalized and recycled back to the cell surface through the late endosomes (Rab-7). We found that IL-7Rα co-localized in the recycling endosomes in the responder more that in the non-responder (57 vs 31% of positive cells), suggesting that in the non-responders the recycling process of internalized receptor didn't occur properly (Figure 4D).


A
+ EEA1
+ IL-7Rα
Merge
Responder
Non-Responder
B
+ Rab-7
+ IL-7Rα
Merge
Responder
Non-Responder
C
Co-localization events on a single cell basis (%)
Responder
Non-Responder
EEA1
D
Co-localization events on a single cell basis (%)
Responder
Non-Responder
Rab-7

Activation markers

We next compared through cytometry the up-regulation of the activation markers, such as HLA-DR, CD25 and CD69 between the most responder and non-responder at all. As shown in Figure 5A, in both patients a high percentage of constitutively activated T cells (CD3+HLADR+) was observed. Moreover, in the non-responder at resting condition, the percentage of CD25+ T cells was higher (22%) than in the responder (1%). After stimulation with PHA, the CD3+CD25+ and CD3+CD69+ cells significantly increased only in the responder (Figure 5A). The increase of the activation markers inversely paralleled the behavior of the expression of CD127, which decreased upon stimulation only in the responder from 85 to 65 % similarly to the control, suggesting a physiological internalization of a functional receptor (Figure 5B).


A
Responder
Non-Responder
CD69+
1%
CD25+
1%
HLA-DR+
11%
CD69+
0.9%
CD25+
22%
HLA-DR+
25%
Unstimulated
CD69+
65%
CD25+
42%
HLA-DR+
38%
CD69+
2.9%
CD25+
18%
HLA-DR+
16%
Stimulated
B
CD3+CD127+ cells (%)
100
75
50
CTR
Responder
NonResponder
Unstimulated
Stimulated

DISCUSSION

In the last few years several studies have documented a positive effect of *in-vivo* administration of steroids on neurological signs of patients affected with A-T. However, this effect was remarkable only in a few patients, while other subjects didn't show any positive response. This variability was only partially explained by the extent of the cerebellar atrophy and redox status and was not correlated to the amount of the residual ATM protein, in that it was absent in all the patients studied. Intriguingly, a similar variability, among the patients receiving the steroid, was also observed in the behavior of the PBMC proliferative response to mitogens during the trial. Thus, by taking advantage of this clinical and immunological variability, in this study we compared several immunological functions, including PBMC proliferative responses, cell activation events and IL-7/IL-7Rα axis functionality, with the neurological behavior during an *in-vivo* steroid treatment between the most responder patient to GC and the non-responder at all.

In this study, we documented a significant reduction before the GC treatment of the proliferative response to PHA and IL-7 in A-T lymphocytes, as expected. IL-7 is a key immunoregulatory cytokine, which plays an essential role in the development and differentiation of T cells (31). The biological effects of IL-7 are mediated via IL-7 receptor complex, and has been shown that IL-7 promotes a negative feedback on its own receptor by downregulating the expression of the IL-7Rα (32), through both suppression of *IL-7Rα* gene transcription and by internalization of existing IL-7Rα protein from the cell membrane (33). The pivotal role of IL-7/IL-7Rα axis is also underlined by the fact that a partial deficiency in this receptor in humans is sufficient to abrogate T-cell development and causes severe combined immunodeficiency (SCID) (34). Intriguingly, we found a significant increase of the proliferative response to PHA or IL-7 of PBMC only in the patients who responded to GC the most at the neurological evaluation, thus indicating a tight correlation between the proliferative responses and the neurological behavior

during the *in-vivo* GC administration. As expected, the proliferative response to IL-7 significantly correlated to the percentage of CD3+ cells expressing the IL-7Rα.

A proper functionality of the IL-7/IL-7Rα axis is based on a continuous turn-over of the receptor through its internalization and recycling. The number of cell surface receptor molecules represents a continuous equilibrium between the rate of synthesis, internalization and recycling of the molecule. The overall process represents a sign of a fully functional receptor turn-over. Upon activation, the decrease of the number of surface receptors on the cell membrane directly reflects their degradation (35).

GCs have pleiotropic effects on the growth, differentiation, and function of lymphocytes (36). Moreover, they may influence either positively or negatively the function of T cells (37). In addition, it has also been reported that IL-7Rα is one of the most prominent gene induced by glucocorticoids and that GCs are able to increase its expression in human peripheral T cells. In this study, we couldn't find any difference between the responder and the non-responder in the IL-7Rα mRNA expression, as compared to the well-known GC gene target, GILZ. This finding was also confirmed at the protein level, in that no difference was observed in the amount of the protein between the responder and the non-responder. Taken together these data would suggest an alteration of the post-transcriptional events in the IL-7Rα biology.

Increasing evidence indicates that endocytosis is a necessary step for efficient receptor mediated signal transduction (37). The presentation of cytokine receptors at the membrane surface, as of other proteins, uses different endocytic pathways, which include clathrin-dependent or independent mechanisms (38). Clathrin-mediated endocytosis requires the assembly of a clathrin coat on the cytoplasmic side of the membrane, becoming free clathrin-coated vesicles. Clathrin-dependent endocytosis delivers internalized cell surface components to early endosomes. Here, molecules that need to be reutilized are recycled back to the surface or are transported to late endosomes for their degradation (39). IL-7Rα is internalized under steady-state and upon IL-7

stimulation through clathrin-mediated endocytosis (40). Additional data demonstrate that the use of inhibitors of clathrin-dependent endocytosis, as nystatin, induce a reduction of the uptake of the receptors in the cells (41). Once internalized, a significant fraction of the receptor is recycled back to the cell surface, which is essential to maintain a proper IL-7Rα surface expression. In this study, we found that in the responders, IL-7Rα is internalized by the cell and abundantly sorted into the early endosome (EEA1 vesicles), differently from the non-responders who had a higher membrane expression of IL-7Rα, and whose vesicles scarcely contained IL-7Rα. Moreover, in the responders a higher amount of IL-7Rα, as compared to the non-responder, is destined for the late endosome (Rab-7 vesicles), indicating a proper recycling of the receptor. Under experimental conditions, in Rab-knockdown cells the lack of Rab is responsible for the accumulation of endocytosed receptors on the membrane surface (42), this feature being very similar to what observed in the cell from the non-responder patients. Taken together, these data would suggest that in the non-responders the overall internalization/recycling process represents a limiting step in cell functionality.

The abnormalities in the IL-7Rα intracellular trafficking reflects a more general deficiency in cell activation, in that the evaluation of the T-cell activation markers, CD69, CD25 and HLA DR, revealed that only in the cells from a responder the activation stimuli up-regulated these molecules, while their up-regulation in a non-responder was negligible. Moreover, under resting conditions cells from the non-responder expressed high levels of CD25 and HLA DR molecules, indicating a constitutive activation of T cells. The observation of a poor proliferation to IL-7 associated with high basal IL-7Rα expression in the non-responder would indicate a reduced sensitivity to the IL-7, presumably, indicating that a normal internalization and turn-over of the molecule is required for a proper receptor functionality.

Recent evidence indicates that ATM is also localized in the cytoplasm, where it is involved in exocytosis, intracellular vesicle and/or protein transport and in the autophagy pathway, whose alterations have been documented also in other neurodegenerative disorders (Alzheimer's,

Parkinson's, Huntington's diseases). During autophagy, the autophagosomes, containing the waste, fuse with the lysosomes resulting in the formation of autolysosomes, where the digestion occurs. Recently, we documented that, in the absence of ATM, an accumulation of waste material within the cytoplasm occurs, indicating that the cell clearance apparatus, involving autophagy and the intracellular vesicle trafficking, is abnormal in A-T (43). Therefore, it is conceivable to hypothesize that the abnormal IL-7Rα trafficking is a more general phenomenon related to the abnormalities of the cell clearance process.

The tight correlation between the immunological improvement during *in-vivo* GC administration and the neurological response to the therapy would suggest that cell biology studies on peripheral lymphocytes are a precious tool to investigate the overall disease pathogenesis and to pave the way to explore innovative therapeutic interventions, able to interfere in the cell biology and eventually in modulating the expressivity of the disease phenotype. Since in all patients studied ATM was undetectable, the clear-cut variability in the neurological and immunological responses would imply that additional genetic or environmental factors are capable to modulate the phenotypic expression of the disease.

The data herein reported have been submitted as *Original article* on *Journal of Clinical and Experimental Immunology*

CHAPTER II

"Characterization of Patients With Increased IgM Levels, B-Cell Differentiation Blockage, Lymphoproliferation and DNA Repair Defect"

The earliest evidence that individuals with PIDs develop cancer was reported in 1963 (44). An increasing number of reports subsequently indicated that subjects with congenital abnormalities of the immune system are at a high risk for developing cancer including lymphoma and stomach, breast, bladder, and cervical epithelial cancers (45)

The overall risk for children with PIDs of developing malignancy is estimated at 4–25% (44, 46). The type of malignancy that is seen is highly dependent on the precise PID, the age of the patient, and probably viral infection indicating that different pathogenic mechanisms may be implicated in each case (46). According to the Immunodeficiency Cancer Registry (ICR) database NHL and Hodgkin's disease (HD) account for 48.6 and 10%, respectively, of the malignancies seen in patients with PIDs. Genomic instability due to defective DNA repair processes and other unknown mechanisms in PID patients leads to an enhanced risk of cancer.

The findings of elevated serum IgM with low IgG, IgA, or IgE in the setting of immune deficiency leads most immunologists toward a diagnosis of Hyper IgM syndrome (HIGM), rare inherited PIDs characterized by class switch recombination defects (CSR) and sometimes impaired somatic hypermutation (SHM). SHM plays a role in the selection and proliferation of B cells expressing a B-cell receptor (BCR) with a high affinity for the antigen and does require the integrity of the cell DNA repair machinery.

The majority of these forms are caused by X-linked (XL) or autosomal recessive (AR) defects in the CD40 ligand/CD40 signaling pathway or AR disorders involving activation-induced cytidine deaminase (AID), or in the uracil DNA glycosylase (UNG). Other HIGM are caused by mutations or in the X-linked nuclear factor k-B essential modulator *(*NEMO*)* gene*,* reported in patients affected with ectodermal dysplasia. In all these cases, both SHM and CSR processes are equally impaired. The unique condition, so far described, of dissociation between the CSR and SHM process is represented by the autosomal dominant mutation in C terminal end of AID in patients showing defective CSR but intact SHM. This observation would imply a different molecular control of the 2 processes. To date, in spite of the identification of new genetic defects associated with HIGM through NGS technologies, in about the 15% of the cases of HIGM patients, the molecular defect still remains to be defined.

The clinical phenotype of HIGM is invariably severe and characterized by increased susceptibility to recurrent bacterial and opportunistic infections, neutropenia, autoimmunity and cancer susceptibility.

The presence of elevated levels of IgM were also reported associated with other immunological defects like RAG2, ATM and ARTEMIS deficiency or to acquired causes, such as in autoimmune diseases, with IgM autoantibodies, or in chronic infections. In B-cell lymphoproliferative disorders, elevated monoclonal IgM levels may also be found.

In this paper, submitted on *Journal of Clinical Immunology*, we report on a group of unrelated patients with very high polyclonal IgM levels, resembling a HIGM of unknown molecular defect, whose clinical course was complicated by the occurrence of a lymphoproliferative disorder. In these patients an evaluation of B-cell subsets has also been performed, revealing a reduction of memory and switched memory B cells. Through the comet alkaline and micronucleus (MN) assays on peripheral blood lymphocyte or fibroblast cultures an increased genotoxicity was documented. In order to evaluated a molecular cause of the disorder NGS analysis was performed, revealing in two subjects mutations in PIK3R1 or ITPKB genes, implicated in B- and T-cell development.

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Elevated polyclonal IgM levels and impaired B cells homeostasis associated to increased susceptibility to lymphoproliferation

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	incidence of lymphoproliferative disorders, who underwent immunological studies, including extended B-cell immunophenotyping, evaluation of class switch recombination and somatic hypermutation, and next generation sequencing. Results An impaired B-cell homeostasis with reduced memory and switched-memory B cells was documented in 4/5 patients studied. Class switch recombination was functional in vivo in all patients as proven by normal specific IgG antibody response. In 2 patients, this finding was also confirmed in vitro, thus ruling out an intrinsic defect in the class switch recombination machinery, although it was associated to a significant impairment of somatic hypermutation. Next generation sequencing revealed in two of them mutations in the PIK3R1 or ITPKB genes, implicated in B- and T-cell development, survival and activity. Conclusion This report gives further insight into the B-lymphocyte disorders, and highlights a possible link between polyclonal hyper-IgM, as a biomarker of immune dysregulation particularly affecting B-cell homeostasis, and susceptibility to lymphoproliferation.
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Elevated polyclonal IgM levels and impaired B cells homeostasis associated to increased susceptibility to lymphoproliferation

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Abstract

Purpose Elevated polyclonal IgM levels represent a hallmark of Hyper IgM syndromes, rare inherited immunodeficiencies characterized by a defect of class switch recombination, sometimes associated to impaired somatic hypermutation. DNA repair defects or common variable immunodeficiency may also associate with a hyper-IgM phenotype. The aim of this study is to highlight a link between immune impairment, characterized by polyclonal hyper IgM and altered B-cell homeostasis, and cancer susceptibility.

Methods We herein report on 6 patients with high polyclonal IgM levels and a high incidence of lymphoproliferative disorders, who underwent immunological studies, including extended B-cell immunophenotyping, evaluation of class switch recombination and somatic hypermutation, and next generation sequencing.

Results An impaired B-cell homeostasis with reduced memory and switched-memory B cells was documented in 4/5 patients studied. Class switch recombination was functional in vivo in all patients as proven by normal specific IgG antibody response. In 2 patients, this finding was also confirmed in vitro, thus ruling out an intrinsic defect in the class switch recombination machinery, although it was associated to a significant impairment of somatic hypermutation. Next generation sequencing revealed in two of them mutations in the *PIK3R1* or *ITPKB* genes, implicated in B- and T-cell development, survival and activity.

Conclusion This report gives further insight into the B-lymphocyte disorders, and highlights a possible link between polyclonal hyper-IgM, as a biomarker of immune dysregulation particularly affecting B-cell homeostasis, and susceptibility to lymphoproliferation.

Key words Hyper-IgM syndrome · Lymphoproliferative disorders · Class switch recombination · Somatic hypermutation · DNA repair

Introduction

High IgM levels may be found in rare immunological disorders, such as hyper IgM syndromes (HIGM), but also in autoimmune or acquired infectious diseases [1-3]. In B-cell lymphoproliferative disorders, elevated IgM levels may be found, but they are monoclonal [4, 5].

HIGM are characterized by normal or increased IgM levels and absent or strongly reduced levels of the other Ig isotypes due to impaired class switch recombination (CSR), which sometimes parallels an abnormal somatic hypermutation (SHM). Both processes require the integrity of the cell DNA repair machinery, although with different repair mechanisms. Within the genetic disorders, 6 genes have been so far implicated, coding for molecules involved in the CD40/CD40 ligand signaling *(CD40L, CD40)*, or in cytosine/cytidine deaminase process *(AID)* [6-8]. Other HIGM are caused by mutations in the uracil DNA glycosylase (*UNG*), in post-meiotic segregation 2 (*PMS2*) and *INO80* genes [9-11]. Overall, the clinical phenotype of HIGM is variably severe and characterized by increased susceptibility to recurrent bacterial and opportunistic infections associated with autoimmunity and cancer susceptibility. Elevated IgM levels were also reported in other well-defined immunodeficiencies as part of heterogeneous clinical phenotypes, like *NEMO, RAG2*, *LRBA*, *ATM*, *ARTEMIS* and *DOCK2* deficiency [3, 12-17]. However, in spite of the introduction into the clinical setting of next generation sequencing (NGS) technologies, allowing a timely genetic diagnose, a few immunodeficiency conditions, including those with elevated IgM levels, still remain to be elucidated in the intimate molecular alteration [1].

We report on 6 unrelated patients with high polyclonal IgM levels, whose clinical course was complicated in 4 cases by the occurrence during the follow-up of a monoclonal lymphoproliferative disorder (LPD). B-cell subsets evaluation revealed a reduction of memory and switched-memory B cells. NGS revealed in two patients mutations in the genes, phosphoinositide-

3-kinase regulatory subunit 1 (*PIK3R1),* and inositol 1,3,4, trisphosphate kinase β *(ITPKB)*, both of them playing a role in promoting B- and T-cell development, survival and activity.

In this study, we highlight a link between immune impairment, characterized by polyclonal hyper IgM and altered B-cell homeostasis, and cancer susceptibility.

Methods

Patients

Six patients (four female) from unrelated non-consanguineous families with high IgM levels (range 416-1060 mg/dl) were enrolled in the study, after signed informed consent. The study was carried out in accordance with the Declaration of Helsinki and approved by the institutional ethics committee.

B-cell immunophenotyping and CD40-CD40L expression

Whole blood anti-coagulated with EDTA was used for multi-color flow cytometry immunophenotyping and processed within 24 h. Cells were exposed to directly conjugated mouse anti-human monoclonal antibodies (mAb) using the following fluorochrome conjugated antibodies: anti-CD45 peridinin-chlorophyll (PerCP) or anti-CD45 horizonV500 (HV500), anti-IgD phycoerythrin (PE), anti-IgM allophycocyanin (APC) and anti-IgG phycoerythrin (PE), all from BD Biosciences (San Jose, CA, USA), anti-CD19 allophycocyanin (APC), anti-CD27 FITC all from Invitrogen/Caltag (Karlsruhe, Germany). The cells were incubated with directly labelled antibody at 4°C in the dark for 30 min, washed and re-suspended in 4 ml of NH_4Cl and then washed in PBS. Flow cytometric analysis was performed on a BD FACS Canto II flow cytometer (BD Biosciences) and analytical flow cytometry was performed using BD FACSDiva software. Lymphocytes were

identified by gating on viable $CD45^+$ cells or identified by gating on FSC and SSC and B-lymphocytes were gated on $CD19^+$ cells. CD40L and CD40 staining were performed through directly conjugated mouse anti-human mAb.

Study of class switch recombination and somatic hypermutation *in vitro*

PBMC were cultured in the presence of 500 ng/ml of soluble CD40-L and 100 U/ml of rIL-4. Proliferation was assessed by measuring [3H] thymidine uptake. IgE production was evaluated in supernatants by ELISA on day 12 [18]. SHM generation in the VH3-23 region of IgM on purified CD19+CD27+ B cells was performed in two patients as previously described [8]. VH3-23 region was chosen since V3-23 Ig V gene is expressed in 4%–10% of B cells [19]. PBMCs were isolated by flow cytometry using FITC-anti-CD19 and PE-anti-CD27 mAb (Immunotech). RNA was purified with the Trizol reagent and cDNA was obtained by reverse transcription with an oligo dT primer. PCR was carried out with the Pfu polymerase (PfuTurbo, Stratagene) using primers for the VH3-23 leader exon (5' GGCTGAGCTGGCTTTTTCTTGTGG-3') and Cμ region (5'-TCACAGGAGACGAGGGGGAA-3') (35 cycles at 94-C for 45 s, 60-C for 1.5 min, 72-C for 2 min). PCR products were analyzed using the TA cloning kit (Invitrogen, San Diego, CA), and VH3-23 positive colonies were sequenced with the dRhodamine dye terminator cycle sequencing kit (Applied Biosystems prism, Foster City, CA) and analyzed with the Applied Biosystems prism 310 genetic analyzer. Frequency of mutations, expressed as percentage of mutated nucleotides/all nucleotides, was evaluated for each clone on 300 nucleotides of the VH3-23 region including CDR1 and CDR2 domains. Polyclonality was established on the basis of the diversity of the CDR3 of B cells bearing IgM.

Whole exome and Sanger sequencing

Whole exome sequencing (WES) was performed in 5 cases (Pt 6 died before), while direct Sanger sequencing of the following genes was performed in selected cases: *CD40LG* (P3-P6), *CD40* (P5, P6), *AICDA* (P1, P4-P6), *UNG* (P1, P5, P6), *NEMO* (P1), *TNFRSF13B* (P1), *ATM* (P1), *PIK3CD* (P1-P5), *PIK3R1* (P1-P5). Genomic DNA was isolated from peripheral blood lymphocytes using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Samples were sequenced to at least 2.5 GB on an Illumina MiSeq with TruSeq MiSeq V3 reagents, yielding paired 250 nucleotide reads. Samples were prepared for exome sequencing using the TruSeq HT library preparation kit (Illumina; San Diego, CA, USA) and subsequent exome enrichment through the xGen Exome Research Panel V1.0 (Integrated DNA Technologies; Coralville, IA, USA) according to manufacturers' protocols. Bioinformatics analysis were carried out, as previously described [20, 21].

Real-time qRT-PCR of *ITPKB* gene

RNA from P3 and controls PBMCs 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. Primer sequences for ITPKB were: Primer A forward: 5′- GCACTGGTCTCCCTTCGTC-3′; reverse: 5′-

CCAGGTAGGTCCTGATTCCC-3′; Primer B forward: 5′- GGACACGCAGGGAGTTTCAAG-3′; reverse: 5′- TCGTCCATCTGGTTGTAGCG-3′; for Actin were: forward: 5′- GATCAAGATCATTGCTCCTCCTG-3′; reverse: 5′- AGGGTGTAAAACGCAGCTCA-3′. Results are mean ± standard error (SE) of 3 repeated 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. Data presented as the means ±SD were analyzed with a Student's t test analysis. P values <0.01 were considered significant.

Results

Clinical and immunological data of HIGM-like patients

As shown in Table 1, five of the 6 patients studied suffered from recurrent bacterial and viral infections, in particular of the upper and lower respiratory tract, causing in P1 a significant lung damage characterized by bronchiectasis and atelectasis. Atelectasis were also found in P4, whereas interstitial lung disease was documented in P2. P1 and P2 also experienced a measles complicated by pneumonia (P1) and a chickenpox with severe ocular involvement (P2). Quantitative PCR for EBV, CMV, HCV-RNA, and HBV-DNA, was negative in patients with hypogammaglobulinemia, who underwent Ig replacement therapy (P1, P4-P6). Involvement of the reticulo-endothelial system with lymphadenopathy, liver and spleen enlargement was documented in 5 patients. Three patients had autoimmunity or signs of chronic inflammation: alopecia (P4), autoimmune myofascitis and recurrent fever (P1, P2). Other clinical features included urticarial-like lesions or other recurrent skin lesions in 4 cases, growth retardation and delayed puberty in 2, mood and behavioral disorders in 2 and Arnold-Chiari malformation in one further patient. In four cases, IgG levels were 2 SD

below mean value of age-matched controls, requiring Ig replacement therapy, as shown in Table 2; IgA isotypes were also below reference values in P1, P5 and P6.

Four patients developed during the follow-up a B-cell LPD. P1 received a diagnosis of non-Hodgkin marginal zone B-cell lymphoma with a cutaneous infiltrate at age of 11 y, thereafter requiring matched related donor haematopoietic stem cell transplantation (HSCT). P2 was diagnosed at 47 y with a low-grade mucosa-associated lymphoid tissue (MALT) lymphoma and Helicobacter pylori infection, whose eradication induced MALT remission. P5 received successful chemotherapy for a cervical Hodgkin lymphoma at 19 y. P6 at 17 y of age was treated for a diffuse large B-cell lymphoma, invading the colonic mucosa, but the patient died from infection, as already reported [22]. In P1 and P2 it was possible a long-term longitudinal evaluation of IgM levels well before the onset of the LPD, as illustrated in Figure 1. Both patients had elevated IgM levels, ranging from 680 and 750 mg/dl in P1 and from 910 and 1060 mg/dl in P2. In both patients, no significant further increase in IgM levels was observed when LPD was diagnosed, while a significant reduction was documented in P1 in the subsequent 4 years post-chemotherapy (range 280-386 mg/dl). In both P5 and P6, elevated IgM levels were documented in the first years of life during an immunological evaluation required for bacterial recurrent infections, and persisted high at the time of the LPD onset. An additional patient with a clinical phenotype characterized by elevated IgM, hypogammaglobulinemia, autoimmune manifestations and LPD was identified but not included into the study since most of the immunological evaluation were not available before the onset of LPD.

In P5 and P6, at the time of the LPD diagnosis HIGM-like phenotype was associated with lymphopenia, predominantly involving the CD4 population. In the remaining patients, no significant abnormalities were found in the T-cell compartment (Table 2).

B-lymphocyte profiling and CD40-CD40L expression

B-lymphocyte phenotyping revealed that the total CD19+ B lymphocytes percentage was reduced in P2, P3, P5 and P6 (5, 2, 4 and 0%, respectively) and normal in the remaining patients, as compared to age-matched reference values [23]. CD19+CD20-IgG+ mature B cells were absent in all patients. Circulating CD19+CD27-IgD+ B cells (naive B cells) were normal or low/normal in all patients investigated (P1-P4), implying a normal central B-cell development. CD19+CD27+IgM+ memory B cells were significantly reduced in all patients studied (range 1.6-5% of CD19+cells), as compared to reference values [24]. As illustrated in the Table 2, a significant reduction of CD19+CD27+IgM- switched memory B cells was also observed in 3 of the 4 patients studied (0 and 2.9% of CD19+cells). Intriguingly, as shown in Figure 2, the patients P1 and P2, who developed LPD, showed the most significant alteration in the switched memory B-cell population. However, these patients exhibited a normal capability to produce specific IgG antibodies. Unfortunately, these data were not available for P5 and P6.

CD40L and CD40 expression was normal in all cases except for P3, in which CD40L expression was reduced. In particular, in the patient stimulated CD4+ cells, CD40L expression was not up-regulated differently from what observed in the control (0.2 *vs* 2.7%) (Figure S1), albeit *CD40LG* gene alterations were ruled out by direct sequencing.

Reduced SHM with normal *in vivo* and *in vitro* CSR

Besides the increased IgM serum levels, 4 patients had IgG specific antibodies as shown in Table 3, indicating a functional *in vivo* CSR, even in hypogammaglobulinemic patients before the start of replacement therapy (P1, P4). Two pts were not evaluated because on Ig replacement therapy. In 2 of them (P1 and P2), the activation of B cells through sCD40L+IL4 confirmed *in vitro* a normal

CSR, at least towards IgE (Table 3). In addition, in these patients a normal B-cell proliferation following the exposure to IL4+sCD40L was also found (data not shown).

Two patients could be studied for SHM, P1 in two different occasions over a 5 yr period, while P2 only once. The percentage of mutated clones among the analyzed CDR3-different clones was slightly reduced in both patients (9 out of 11, and 6 out of 10 in P1, and 6 out of 7 in P2- as compared to controls in which nearly all clones were found mutated). But strikingly, the frequency of mutations found in the VH3-23 region was lower than controls (P1: 1.9 and 1.2%, P2: 2.3% versus 3.5-6.3% of controls). However, in spite of the reduced number of mutations, the nucleotide substitution pattern was found normal (Figure 3).

Next Generation Sequencing

Recently, mutations in the *PIK3CD* or *PIK3R1* genes have been reported in novel forms of primary immunodeficiency, characterized by elevated IgM, normal/low IgG levels, lymphopenia, respiratory infections, lymph node enlargement and elevated risk of lymphomas [25-29]. Sanger sequencing of these genes resulted normal in the 4 cases studied (P1, P2, P3, P4). WES revealed in P5 a *de novo* splice site mutation in *PIK3R1* (c.1425+1G>T), already reported [27, 28], and in P3 a heterozygous frameshift mutation in *ITPKB* gene (c.146_147insA), an unreported variant, predicted to damage protein function with high confidence (Figure 4).

Real-time qRT-PCR of *ITPKB* gene from RNA

Quantitative RT-PCR (qRT-PCR) from PBMCs showed that *ITPKB* gene expression was significantly lower in the patient as compared to controls, as illustrated in Figure 5.

Discussion

Here, we report on a case series characterized by a condition of immune alterations with very high polyclonal IgM levels, but normal CSR, associated with high incidence of B-cell tumors, alterations of memory B-cell subsets and reduced SHM. Elevated IgM levels were not directly related to LPD and preceded for a long time the onset of LPD. In spite of a few similarities with genetically determined HIGM, the immunological and molecular evaluation of our case series recalls an entity distinct from known HIGM. In order to better define this immune defect**,** we first studied the B-cell compartment through an extended B-cell immunophenotyping. The analysis revealed an alteration of the B-cell maturation process, characterized by a reduction of memory and class-switched memory B cells, suggesting an altered germinal center function. Notably, there was a significantly more pronounced defect of the CD19+CD27+IgM- switched memory subset in patients who developed LPD. These B-cell abnormalities have also been reported in patients affected with CVID [30, 31]. Of note, CVID patients are also prone to develop lymphomas [32]. Indeed, a significant correlation between high serum IgM levels at diagnosis and the eventual development of either polyclonal lymphocytic infiltration or lymphoid malignancy in CVID patients was reported [33]. However, even though a few patients along with elevated IgM also showed low IgG levels, in none of the 6 patients the CVID criteria were fulfilled (ESID diagnostic criteria). A combined reduction of both unswitched and switched memory B cells was also reported in other forms of primary immunodeficiency, including the X-linked lymphoproliferative syndrome type 1, the Warts, Hypogammaglobulinemia, Infections, Myelokathexis (WHIM) syndrome, or the DOCK8 deficiency [34-36].

We further investigated the CSR and SHM of IgM on CD19+CD27+ B cells. CSR and SHM represent the two major maturation events required for an efficient humoral response, and both take place simultaneously in the germinal center after CD40 activation. Intriguingly, differently from

classical HIGM, we observed a normal *in vivo* and *in vitro* CSR, but a reduced frequency of SHM in the two tested patients. The decrease of SHM could be questionable because of the low numbers of studied clones (due in part to the technique used and the decreased numbers of CD27+ cells), but it was confirmed twice in P1, suggesting an actual defective SHM process. These findings would favor the hypothesis of an underlying altered germinal center functionality, as previously suggested by the reduced memory and class-switched memory B cells. Unfortunately, because of chemotherapy, HSCT or death, we were not able to test more patients for SHM. Nevertheless, we cannot definitively exclude a T-cell defect as causative of this immune alteration, even if 4 out of the 6 patients had no obviously abnormal T-cell phenotype. Decreased numbers of B cells, especially of memory B cells, could also suggest a defective B-cell proliferation or survival. However, the observation of B-cell tumors suggests rather an intrinsic B-cell defect, likely associated to a DNA repair defect. DNA repair defects are known to present with a hyper IgM phenotype, as Non-Homologous-End-Joining (NHEJ) defects or Ataxia-telangiectasia (A-T) [37] and Nijmegen breakage syndromes (NBS) [38], characterized by defective in vitro CSR and normal frequency and pattern of SHM. Defects in base excision or mismatch repair mechanisms lead to variably defective in vitro CSR contrasting with normal frequency of SHM, even if in UNG- and MSH6-deficiencies [9, 39], SHM present with a dramatically abnormal pattern of nucleotide substitution, a phenotype not observed in the herein described patients.

As matter of fact, the high incidence of LPD occurrence in this condition seems to be the hallmark of this disorder, that along with the impaired SHM, reinforce the hypothesis of the presence of increased susceptibility to DNA damage related to a DNA repair deficiency. However, it should be mentioned that the predisposition of PID patients to cancer could also be due to the immunodeficiency itself, as tumor immune surveillance becomes impaired and infections by potentially oncogenic viruses are less likely to be dealt with efficacy [40]. In the last years, several

PID-associated DNA repair proteins have been described, mostly of them affecting one or more processes among V(D)J recombination, CSR and SHM mechanisms. Similarly to our cohort, also in patients with alterations of proteins acting downstream of AID, such as ATM and NIBRIN, or in patients with Artemis or mismatched repair enzymes (MMR) deficiencies, high IgM levels and increased cancer susceptibility have been reported [41, 42], indicating that several pathways are implicated in protecting the cell from genotoxic damage.

Our case series, was finally genetically analyzed firstly through direct sequencing of genes expected to be involved on the basis of clinical and immunological phenotype, and subsequently through next generation sequencing, since no genetic alteration was found using the classic diagnostic approach. WES allowed to identify a variation in 2 genes implicated in the B- and T-cell development and function, *PIK3R1* and *ITPKB*. Recently, heterozygous gain-of-function mutations in *PIK3R1*, encoding for p85α, one of the catalytic subunits of the PI3 Kinase molecules, were reported as responsible for a novel form of immunodeficiency [27-29]. This novel immunodeficiency, similarly to activated PI3 kinase delta syndrome (APDS) due to mutations of another subunits of the PI3K pathway, p110δ, is characterized by elevated IgM and low IgG serum levels, recurrent respiratory infections, lymph node enlargement, poor growth, and elevated risk to develop lymphomas. The *ITPKB* gene variation has never been reported previously, even though a deletion involving this gene has been recently associated with a CVID phenotype with mood disorders [43]. Similarly to the CVID patient described by Luis et al., our patient showed psychiatric disorders and lymphopenia associated with recurrent otitis, without any infectious or eczematous skin lesions and recurrent sepsis. Nevertheless, in our case, the causal relationship between the phenotype and the genetic variation requires an ad hoc study for a formal demonstration. However, we tested the RNA expression through quantitative RT-PCR, which documented a reduction of the RNA expression compared to controls, suggestive of a potential

causative role of the gene. This is in keeping with the recent evidence in murine models documenting its role in T- and B-cell development, function and survival [44, 45].

Conclusions

Taken together, our findings suggest that elevated polyclonal IgM levels with a normal CSR recombination, may be a warning sign for a B-cell disorder and should prompt clinicians to consider a LPD in the investigation of patients affected. Moreover, an in-depth characterization of such patients at molecular and functional level may lead to the identification of novel immunological pathways, paving the way to targeted therapy.

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Authorship Contributions

V.G., E.C., C.P. organized, collected and analyzed the data; V.G., C.P. wrote the manuscript; G.G., R.P., L.d.V., G.S., Al.L., An.L., A.D., performed the experiments; V.Ma., V.Mo., contributed with samples from some patients; G.DM., C.S., performed whole exome sequencing; all authors reviewed and approved the manuscript.

Compliance with Ethical Standards

All procedures were performed upon informed consent and assent from patients, first-degree relatives, and healthy donor controls in accordance with the ethical standards of the institutional and/or national research committees and with the current update of the Declaration of Helsinki.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Clinical features of the HIGM-like patients

	P1	P2	P3	P4	P5	P6†
Age (y)	16	49	45	8	15	15
Sex	F	F	M	M	F	F
Clinical features						
Infections						
Bacterial	+	+	-	+	+	+
Opportunistic	-	-	-	-	-	-
Viral	+*	+**	-	-	-	-
Lung disease						
Bronchiectasis	+	-	-	-	-	-
Atelectasis	+	-	-	+	-	-
Interstitial lung disease	-	+	-	-	-	-
Lymphadenopathy	+	-	+/-	+	+	++
Autoimmunity	-	-	-	+	-	-
Inflammatory disease	-	+	-	-	-	-
Recurrent fever	-	+	-	-	-	-
Musculoskeletal involvement	+	+	-	-	-	-
Liver and/or spleen enlargement	+	-	+	+	-	-
Cutaneous manifestations	+	+	+	+	-	-
Cancer	NH-Lymphoma	MALT-Lymphoma	-	-	Hodgkin-Lymphoma	Diffuse large B-cell Lymphoma
Other			Mood disorder	Behavioral disorder	Growth and pubertal delay; bone defects; Arnold Chiari syndrome	Growth and pubertal delay

NH, non-Hodgkin; *MALT,* mucosa-associated lymphoid tissue;
*P1 experienced a measles complicated by pneumonia
**P2 experienced a chickenpox with severe ocular involvement
† Patient died

Table 2 Immunological and molecular findings of the HIGM-like patients

	P1		P2		P3	P4	P5	P6†
Age (y)	10 Before LPD	16 After LPD	34 Before LPD	49 After LPD	45	8	15 Before LPD	15 Before LPD
Immunological features								
IgG, mg/dl	442↓ *(IVIG)*	660	858	788	1500	294↓ *(IVIG)*	565↓ *(IVIG)*	140↓ *(IVIG)*
IgA, mg/dl	19↓	8.3↓	282	332	227	33	5↓	5↓
IgM, mg/dl	753↑	416↑	911↑	1060↑	516↑	800↑	443↑	596↑
Lymphocyte absolute counts/ml	7.860	6.950	*NK*	3.300	1.400	3.090	2100	2260
T-cell subsets								
CD3+ (% of lympho) (absolute value)	67 (5.266)	72 (5.004)	*NK*	79 (2.607)	86 (1.204)	74 (2.286)	90 (1.890)	95 (2.147)
CD4+ (% of lympho)	23 (1.807)	31 (2.154)	*NK*	57 (1.881)	48 (672)	40 (1.236)	26↓ (546)	14↓ (300)
CD8+ (% of lympho)	40 (3.144)	31 (2.154)	*NK*	21 (693)	36 (504)	30 (927)	62↑ (1.302)	70↑ (1502)
CD56+ (% of lympho)	2.7 (212)	4 (278)	*NK*	14 (462)	6 (84)	6 (185)	*NK*	*NK*
B-cell subsets								
CD19+ (% of lympho)	28.5↑ (2.240↑)	25 (1.737)	*NK*	5↓ (165↓)	2↓ (28↓)	12 (370)	4↓ (84↓)	0↓ (0)
CD19+CD27+IgM+ (IgM memory, % of CD19+)	12.3 (275)	3↓ (52.0↓)	*NK*	20 (33↓)	5↓ (1.4↓)	1.6↓ (5.9↓)	*NK*	*NK*
CD19+CD27+IgM- (switched memory, % of CD19+)	0↓ (0)	2.9↓ (50.3↓)	*NK*	0↓ (0)	10 (2.8↓)	12.5 (46.2)	*NK*	*NK*
Genetic alteration					*ITPKB* c.146_147insA		*PIK3R1* c.1425+1G>T	
Inheritance					*NN*		*de novo*	

NK, not known

Table 3 *In vivo* and *in vitro* Class Switch Recombination

	P1	P2	P3	P4
***In vivo* CSR**				
IgG anti-HbsAg	-	*ND*	-	-
IgG anti-Measles	+	+	*ND*	*ND*
IgG anti-CMV	+	+	+	+
IgG anti-EBV	+	+	+	+
IgG anti-VZV	+	+	+	+
IgG anti-Rubella	-	*ND*	+	*ND*
IgG anti-Mumps	-	*ND*		+
***In vitro* CSR**				
IgE pg/ml (not stimulated)	173	2860	*ND*	*ND*
IgE pg/ml (stimulated)	14658	6120	*ND*	*ND*

ND, not done

Figure Legends

Fig. 1 Long-term serum Ig levels evaluation. A long-term evaluation documented elevated IgM levels 5 and 10 years before the LPD in P1 **(A)**, and in P2, respectively **(B)**. IgG decreased in P1 post-chemotherapy. Age-matched reference values in mg/dl (9-11 y): IgG 707-1919, IgA 60-270, IgM 61-276; (12-16 y) IgG 604-1909, IgA 61-301, IgM 59-297; (>18 y): IgG 737-1607, IgA 70-400, IgM 40-230.

Fig. 2 B-cell immunophenotyping. Representative flow cytometric plot showing total CD19+ cells (left panels), the memory (CD19+ CD27+IgM+) and switched memory (CD19+ CD27+IgM-) B cell populations in patients P1 and P2 (right panels), expressed as percentage of total lymphocyte.

Fig. 3 SHM results on the VH3-23 region of IgM on CD19+CD27+ isolated B cells. **(A)** Frequency of mutations on IgM+CD27+ B cells purified from controls and patients. Dots represent results for each subject. Controls: Black dots: all 10 clones mutated; white dots: 9/10 clones mutated. Patients: P1: *, P2: #. **(B)** Nucleotide substitution pattern for P1 (1st evaluation), P2 and control. The same pattern was observed in P1 in the 2nd evaluation. **(C)** The numbers of mutated clones from all studied clones is shown, as well as the frequency of mutations.

Fig. 4 *PIK3R1* and *ITPKB* variations identified through whole exome sequencing. **(A)** *PIK3R1* gene structure which encodes p85α protein. The splice site mutation in patient 6 is indicated. **(B)** Pedigree of the family carrying the PIK3R1 mutation. **(C)** Sequencing chromatograms in patient and her parents. **(D)** *ITPKB* gene structure with the frameshift mutation identified in P4. Chromosome location and genomic coordinates are provided.

Fig. 5 Real-time qRT-PCR of *ITPKB* gene mRNA expression. Quantitative RT-PCR was performed using two specific couples of primers. ITPKB mRNA expression was normalized on Actin mRNA content. The error bars represent technical replicates within a single experiment. Each experiment has been repeated three times. Statistical analysis was performed by comparison between controls and affected ITPKB mRNA content. *, $p<0.0004$; ns= no statistically significant.

Figure 1

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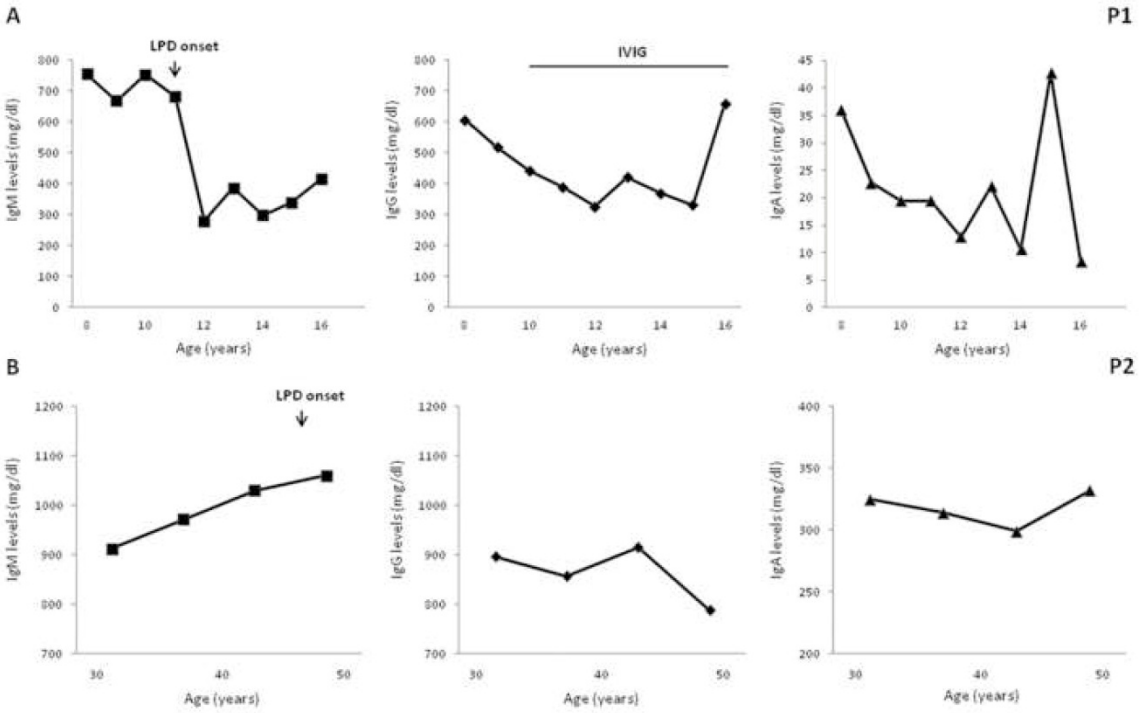

Figure 2

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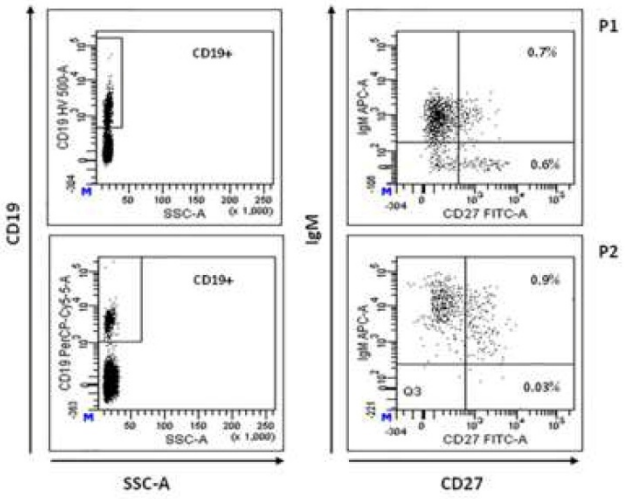

Figure 3

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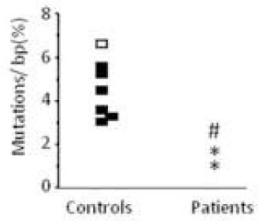
A



B

P1	A	C	G	T	%
A		2	13	3	18
C	0		8	11	19
G	25	11		7	43
T	0	5	15		20
%	25	18	36	21	100

P2	A	C	G	T	%
A		7	5	4	16
C	0		2	17	19
G	33	18		2	53
T	6	6	0		12
%	39	31	7	23	100

CTR	A	C	G	T	%
A		7	9	4	20
C	3		4	12	19
G	29	13		5	47
T	5	6	3		14
%	37	26	16	21	100

C

	Mutated clones	Freq. Mutations %
P1	9/11	1.9
P2	6/7	2.3
CTR	10/10	5.1

Figure 4

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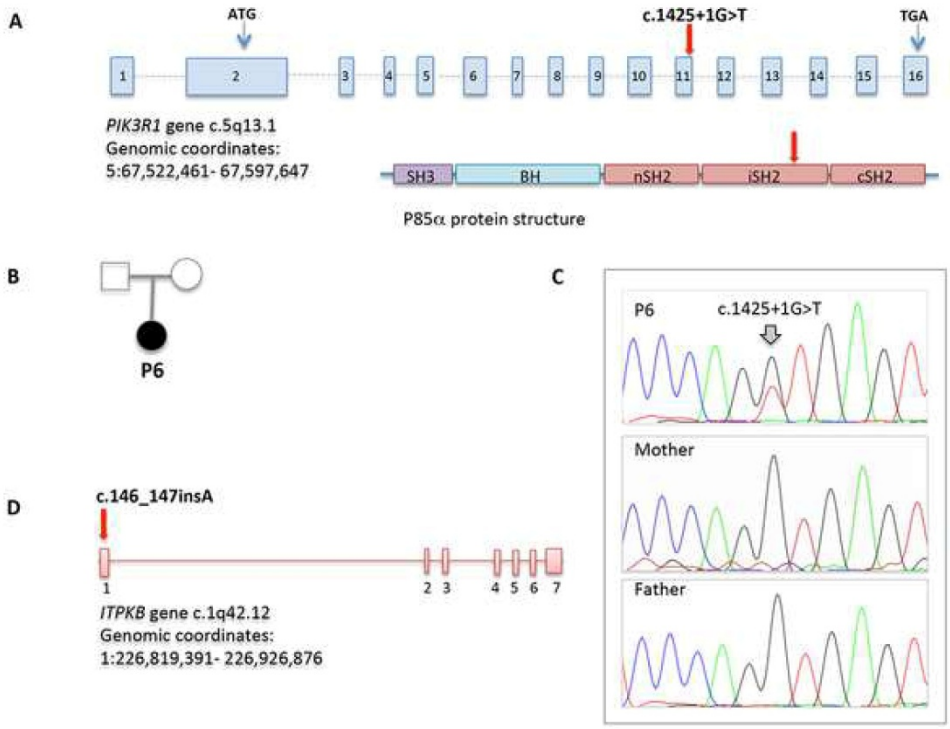

Figure 5

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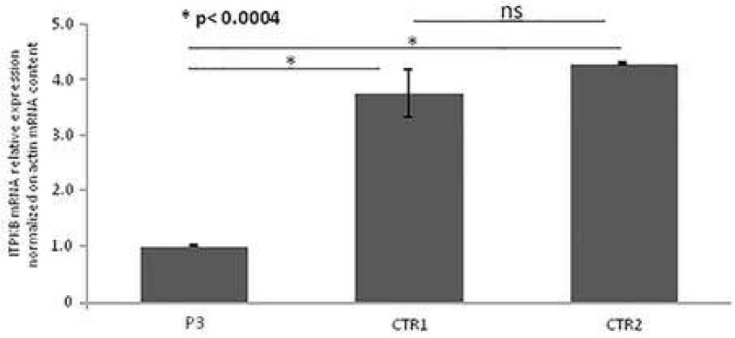


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CHAPTER III

“New insights in the pathogenesis of Ataxia-Telangiectasia disorders”

Ataxia-Telangiectasia clinical hallmark is represented by the cerebellar neurodegeneration with the loss of Purkinje cells (20). Recently, in a few clinical trials it 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 this paper, submitted on *Clinical Immunology*, we indicate that in cells from A-T patients, the APs maturation is active, while the fusion between APs and lysosomes is inappropriate, thus implying abnormalities in the cell-clearance process. We also documented a positive effect of Betamethasone on molecules implicated in autophagosome degradation.

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Abnormal cell-clearance and accumulation of autophagic vesicles in lymphocytes from patients affected with Ataxia-Teleangiectasia

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ABSTRACT

Ataxia-Teleangiectasia (A-T) is a neurodegenerative disorder due to mutations in ATM gene. ATM in the nucleus ensures DNA repair, while its role in the cytosol is still poorly clarified. Abnormal autophagy has been documented in other neurodegenerative disorders, thus we evaluated whether alteration in this process may be involved in the pathogenesis of A-T by analyzing the autophagic vesicles and the genes implicated in the different stages of autophagy. Through transmission electron microscopy (TEM) and immunofluorescence analysis we observed an accumulation of APs associated with a LC3 puncta pattern, and a reduced number of ALs. We also documented an increased expression of genes involved in AP and lysosome biogenesis and function, and a decrease of *Vps18* expression, involved in their vesicular trafficking and fusion. mTORC1-controlled proteins were hyperphosphorylated in A-T, in keeping with an increased mTOR inhibitory influence of autophagy. Betamethasone is able to promote the degradation of SQSTM1, a biomarker of autophagy. Collectively, our results indicate that in cells from A-T patients, the APs maturation is active, while the fusion between APs and lysosomes is inappropriate, thus implying abnormalities in the cell-clearance process. We also documented a positive effect of Betamethasone on molecules implicated in autophagosome degradation.

1. Introduction

Ataxia-Teleangiectasia (A-T) (MIM #208900) is a rare recessive disorder characterized by cerebellar neurodegeneration with the loss of Purkinje cells, oculocutaneous teleangiectasia, susceptibility to cancer, growth retardation, diabetes mellitus and immunodeficiency [1–3]. Recently, in a few clinical trials it has been documented that a short-term treatment with glucocorticoids (GCs) is able to partially rescue the A-T neurological phenotype and lymphocyte proliferation even though the mechanism of action has not yet been defined [4–7]. The improvement of cerebellar symptoms during steroid treatment was inversely correlated with the severity of cerebellar atrophy [6]. It has recently been documented that Dexamethasone *in vitro* leads to the skipping of mutations upstream of nucleotide residue 8450 of *Ataxia Telangiectasia Mutated* (*ATM*) coding sequence, resulting in a new ATM variant with the complete kinase domain, which was shown to be likely active [8].

A-T is caused by mutations in the *ATM* gene encoding a serine/threonine protein kinase involved in cell cycle control and repair of DNA double-strand breaks [9,10]. Evidence exists that ATM exerts additional functions in the cytoplasm independent of its role in the DNA damage response [11], such as participation in the autophagy pathway [12]. 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.

To date, A-T remains an incurable disease that leads relentlessly to death around the third decade of life [13].

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 [14], whose hallmarks are organelle damage, synaptic dysfunction and neuronal degeneration. 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. 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)

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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 [15]. 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) [16]. AMPK regulates several metabolic processes and activates Tuberous sclerosis complex 2 (TSC2), which participates in energy sensing and growth factor signaling [17]. TSC2, by inhibiting the GTPase Ras homolog enriched in brain (Rheb), is able to repress the mechanistic target of rapamycin (mTOR) kinase, a key regulator of the protein synthesis and cell growth, thus leading to the activation of autophagy [18]. 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 the microtubule-associated protein 1 light chain 3 (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 electron 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 [19]. An increase of LC3-II form may result from either an enhancement of AP biogenesis or inhibition of AP degradation, or may be due by other mechanisms [19]. 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 [20]. Thus, increased LC3-II and decreased SQSTM1 levels are indicative of an appropriate autophagic activity whereas increased SQSTM1 levels reveal defective autophagy [20].

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 an autophagic process [21] during catabolic conditions. In particular, the expression of the UV Radiation Resistance Associated (*UVRAG*) gene plays a pivotal role in the first phase of the process, which consists in AP biogenesis and formation [22,23]. 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 (GBA), β-glucuronidase (GUS) and cathepsins (CST) [21]. 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 [24]. Moreover, Vacuolar Protein Sorting 11 and 18 (*Vps11* and *Vps18*) protein products are also involved in vesicular trafficking to allow the encounter between APs and lysosomes, resulting in their fusion [25]. Eventually, the formation of autolysosomes (ALs), whose content is degraded by lysosomal enzymes and recycled, leads to preserved 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.

Through *in vitro* experiments using freshly isolated lymphocytes from A-T patients, we found that in A-T lymphocytes, under resting conditions, there is an accumulation of APs, associated with a high expression of genes involved in the process of biogenesis and function of APs and lysosomes. The addition *in vitro* of Betamethasone helps decrease SQSTM1 levels, which is associated to autophagy progression. Moreover, a reduced expression of *Vps18* and *Vps11* molecules, involved in the vesicular trafficking and fusion between APs and lysosomes, was also found. Taken together, our results indicate that the stage of fusion between APs and lysosomes represents a limiting step, which leads to AP accumulation and inappropriate cell clearance and trafficking in A-T.

2. Materials and methods

2.1. Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from A-T patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation. Lymphoblastoid B-cell lines (BCLs) were generated by Epstein Barr Virus (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% CO_2. Serum starvation was induced incubating the cells in medium without FBS for 2 h. All the experiments were approved by the Ethical Committee "Comitato Etico per le Attività Biomediche Carlo Romano" of the Federico II University of Naples.

2.2. Transmission electron microscopy

PBMCs obtained from A-T patients and healthy donors were washed in PBS and centrifuged to obtain a visible pellet. Cells were than fixed with a 1% glutaraldehyde and 0.2 M 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 ultrastructural 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 [26].

2.3. Western blotting

Total lysates were obtained from lymphoblastoid B-cell lines of A-T patients and healthy donors. The cells were pre-treated or not with 80 nM of Betamethasone (BMZ) for 2 h, 0.2 mM H_2O_2 for 30 min, or 100 nM Bafilomycin A1 (BAFA1) (Sigma-Aldrich, St. Louis, MO) for 1 h, 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 (Na_3VO_4), 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 PBMCs from A-T patients and healthy donors. The cells were treated with 0.2 mM H_2O_2 for 15, 30 or 60 min, washed with ice-cold PBS and resuspended in Buffer A (10 mM Hepes/KOH pH 7.9, 1.5 mM $MgCl_2$, 10 m MKCl, 0.5 mM Dithiothreitol (DTT) pH 7.9, 0.5 mM PMSF, 0.5 mM Na_3VO_4, 5 μg/ml leupeptin and 5 μg/ml aprotinin) on ice for 10 min. 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, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM Na_3VO_4, 5 μg/ml leupeptin and 5 μg/ml aprotinin) on ice for 20 min. 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% Novex NuPAGE 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 antibodies for LC3 (Biorbyt, Cambridge, UK), SQSTM1 (Fitzgerald Industries International, MA, USA), p-S6K (Thr389) (Cell Signaling Technology), p-S6 (Ser 235/236) (Cell Signaling Technology), 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 antibodies. ECL reagent (Bio-Rad, Woodinville, WA, USA) was used as detection system for visualization. Densitometric analysis was performed using ImageJ software.

2.4. Real-time quantitative reverse transcriptase PCR analysis

Total RNA was extracted from ATM-deficient and control PBMCs 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 Cycler 480 (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 1. Results are mean ± standard error (SE) of 2 repeated 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 [27].

2.5. Fluorescence microscopy

PBMCs obtained from A-T 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 a mix of 1:100 LC3 (Biorbyt, Cambridge, UK) and 1:100 lysosome-associated membrane protein (LAMP) (Biorbyt, Cambridge, UK) antibodies. Appropriate anti-rabbit or anti-mouse peroxidase-linked secondary antibodies 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).

2.6. Statistics

GraphPad Prism software was used for data analysis. The Student's *t*-test was used to analyze the statistical significance of differences. The minimum acceptable level of significance was $p \leq 0.05$ calculated through two-tailed unpaired Student's *t*-test and CHI Test.

3. Results

3.1. Lymphocytes from patients affected with A-T show accumulation of APs

To address the involvement of an 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 (Fig. 1A) and the accumulation of autophagic vesicles (AVs) at different maturation stages (*i.e.* APs and ALs) in a cell after serum starvation for 2 h (Fig. 1B). Ultrastructural analysis of lymphocytes from A-T patients at basal conditions revealed a general increase in AV size and number (Fig. 1C–F) not observed in healthy control cells.

Next we analyzed distinct types of AVs, such as APs and ALs. TEM revealed that A-T patients exhibit a higher number of APs and a 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) (Fig. 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 negative fields, respectively; $p < 0.01$) (Fig. 1H), resulting in an APs/ALs ratio much higher in the

Table 1
Sequences of oligos used in Real-Time qPCR analysis.

Gene symbol	Gene name	Sequence (5′–3′)	
		Forward primer	Reverse primer
UVRAG	UV Radiation Resistance Associated	TGACAATTCGTTGCAGGCAGTTA	AGGCAACTTGACACCGCATACA
VPS11	Vacuolar Protein Sorting 11	CCACTTTGATGTGGAGACAGC	TGTATCGAAGGGCTTCCTGA
VPS 18	Vacuolar Protein Sorting 18	AGCGTCGCTACCTGGAGAG	GTACGTTCGGCTGGCTTC
CSTF	Cathepsin F	ACAGAGGAGGAGTTCCGCACTA	GCTTGCTTCATCTTGTTGCCA
CSTB	Cathepsin B	AGTGGAGAATGGCACACCCTA	AAGAAGCCATTGTCACCCCA
CSTD	Cathepsin D	AACTGCTGGACATCGCTTGCT	CATTCTTCACGTAGGTGCTGGA
CSTA	Cathepsin A	CAGGCTTTGGTCTTCTCTCCA	TCACGCATTCCAGGTCTTTG
CSTE	Cathepsin E	GGACATGATCCAGTTCACCGA	GGTTGGCACACTCCACAGCA
GBA	Glucosidase, beta, acid	TGGGTACCCGGATGATGTTA	AGATGCTGCTGCTCTCAACA
GUS	Glucoronidase	ATTGGAGGTGCAGCTGACTG	TCCTCATGCTTGTTGACACC
CD63	CD63 molecule	TCACGTTTGCCATCTTTCTG	TCGAAGCAGTGTGGTTGTTT
KIF1Bβ	kinesin family member 1B	GACCAAGCTCAGTGGTCTCTAAG	CCAGATGACCTGGTCGTATTGTGC
KIF2A	kinesin heavy chain member 2A	GCCTTTGATGACTCAGCTCC	TTCCTGAAAAGTCACCACCC
ARL8A	ADP-ribosylation factor-like 8A	AGTCCTGGGTAACAAGCGAGAC	GCAAGAGATGGAGTAGCAGCAG
ARL8B	ADP-ribosylation factor-like 8B	CACCTTCGTCAATGTCATCG	CCTATGTCCCAGATCTTTATTGTG
KIF3C	kinesin family member 3C	GAAGATGCTGGAGGACCTGCGG	GTAGGTGCCCCGGAGCTCCATAG
β-actin		GACAGGATGCAGAAGGAGAT	TTGCTGATCCACATCTGCTG

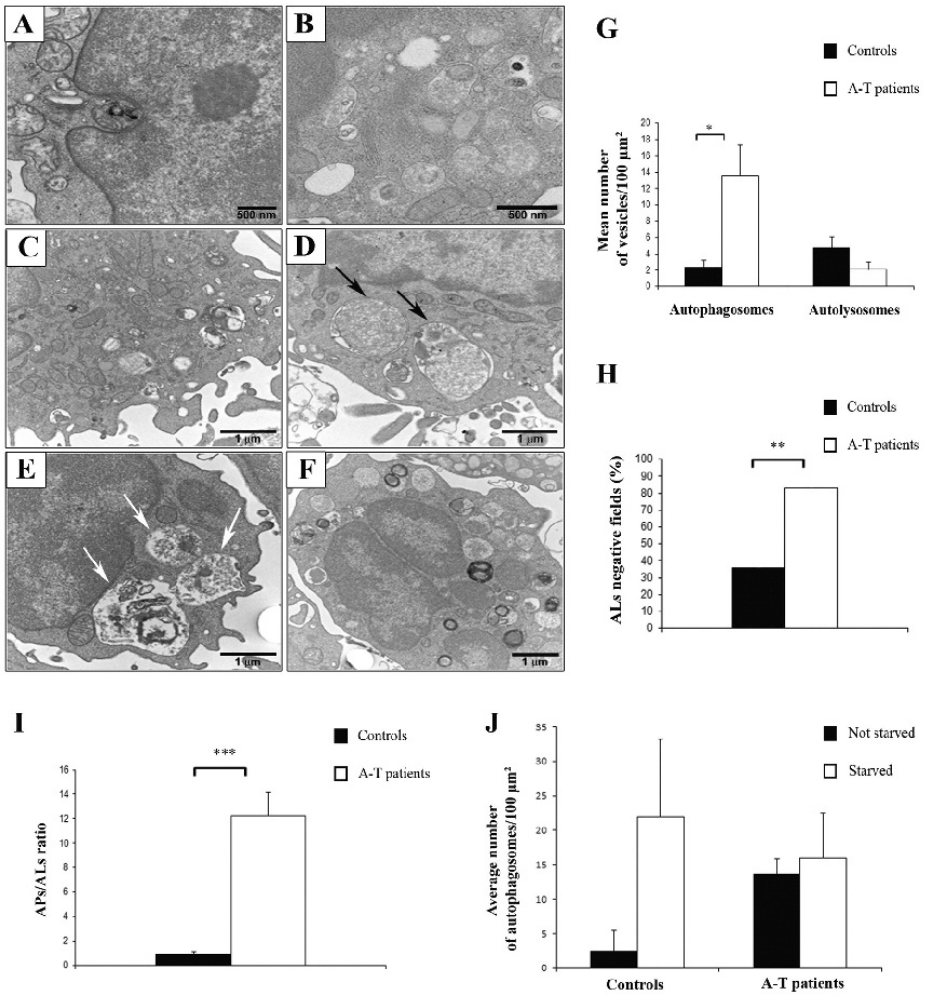

Fig. 1. Lymphocytes from patients affected with A-T show accumulation of autophagosomes. (A–B) Electron micrographs of freshly isolated lymphocytes from healthy controls in unstarved culture conditions or after serum starvation for 2 h showing the accumulation of autophagic vesicles (AVs). Scale bar: 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. (H) Percentage of AL negative fields in fresh isolated lymphocytes from healthy controls and A-T patients. (I) APs/ALs ratio for freshly isolated lymphocytes from healthy controls and A-T patients. (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 2 h. (Data expressed as mean ± S.D.; $^*p = 0.03$; $^{**}p < 0.01$; $^{***}p < 0.05$. Representative image, $n = 3$ subjects per group).

patients than in the controls (12.22 *vs* 0.93; $p = 0.03$) (Fig. 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 AP biogenesis occurs (Fig. 1J).

The formation of LC3 puncta is observed in cells during autophagic activation [19]. 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 (Fig. 2A). Moreover, as shown in Fig. 2A, in few lymphocytes from A-T patients the LC3 marker co-localized with LAMP2, in contrast to the controls, where no merge signal between LC3 and LAMP2 was documented. This result indicates that in the patients, even at basal conditions, autophagy was detectable, since the presence of the merge signal indicates that the fusion between APs and lysosomes occurred. 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%) (Fig. 2B) and that in A-T patients about 4/5 of the total amount of LC3 molecules contained in the cells showed the puncta staining pattern (82% puncta *vs* 18% non-puncta; $^*p < 0.005$), 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) (Fig. 2B).

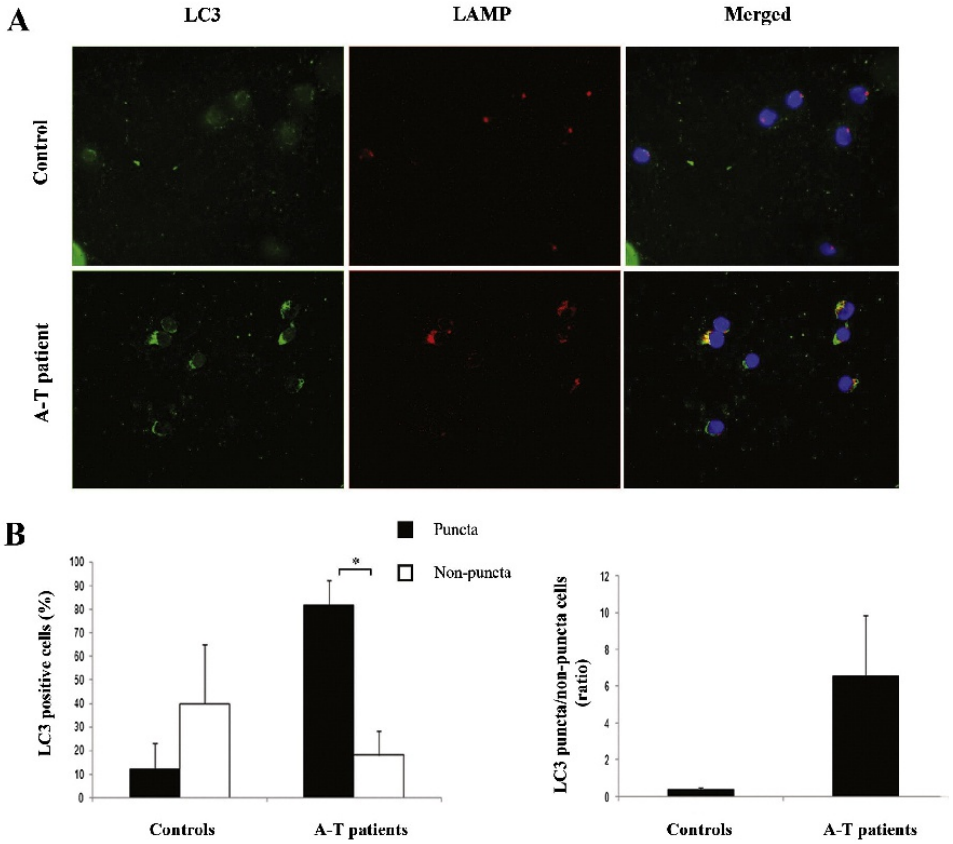

Fig. 2. Autophagy is active in lymphocytes from A-T patients at basal conditions. (A) Representative images of freshly isolated lymphocytes from healthy controls and A-T patients showing autophagosomes expressing LC3 marker (green punctate dots), lysosomes expressing LAMP2 marker (red dots) and autolysosomes (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.; $^{*}p < 0.005$; representative image, $n = 4$ subjects per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. The accumulation of APs in lymphocytes from patients affected with A-T is associated to a reduced expression level of Vps18 mRNA

Autophagy consists of two consecutive stages: the early stage when the APs biogenesis occurs and the late stage characterized by the fusion between APs and endosomes-lysosomes, which leads to the formation of ALs, where the damaged proteins and the entire organelles are degraded by the lysosomal hydrolases and recycled to generate building blocks for anabolic processes. Of note, a normal biogenesis and function of lysosomes and their positioning in the cell, are essential for a proper autophagic process. Since several genes are activated during the process, 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 (Fig. 3A), in keeping with the data obtained by TEM. The evaluation of the mRNA expression level of genes involved in the lysosome 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 (Fig. 3B), while under starvation conditions the expression was comparable (data not shown), 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 (Fig. 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 Fig. 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 Fig. 3D, at basal conditions, all the kinesins, and in particular the *KIF3C* molecule, were more expressed in A-T

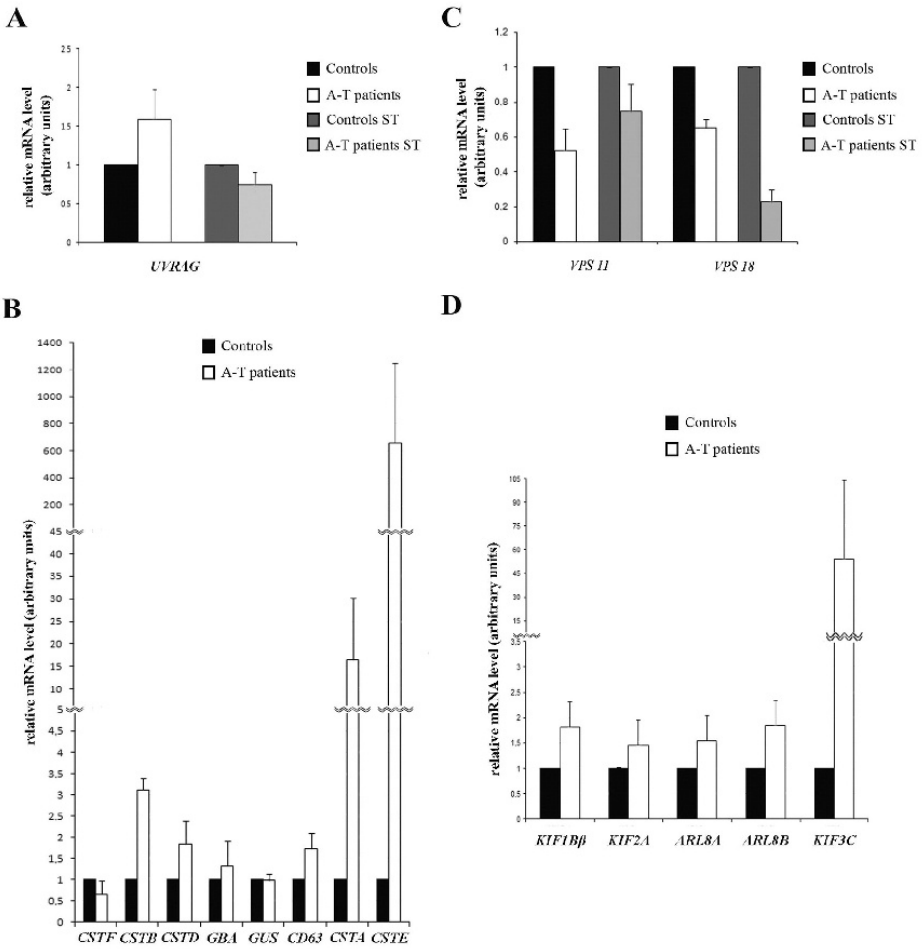

Fig. 3. The accumulation of autophagosomes in lymphocytes from A-T patients is associated to a reduced expression level of Vps18 mRNA. (A–B) Relative mRNA expression level of genes involved in the biogenesis of APs (*UVRAG*) and in lysosome biogenesis and function (*CSTF*, *CSTB*, *CSTD*, *GBA*, *GUS*, *CD63*, *CSTA*, *CSTE*), in freshly isolated lymphocytes from healthy controls and A-T patients, at basal conditions and after serum starvation for 2 h. (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 2 h, and in lysosomal positioning in the cell (*KIF1Bβ*, *KIF2A*, *ARL8A*, *ARL8B* and *KIF3C*) in freshly isolated lymphocytes from healthy controls and A-T patients, at basal conditions (data are expressed as mean ± S.E.; $n = 3$ subjects per group).

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.

3.3. Autophagy is increased in A-T patients despite an activated mTOR pathway

mTORC1 is an important sensor in regulating cell proliferation and inhibiting autophagy [28]. Several pathways regulate its activation, which eventually results in the phosphorylation of downstream targets S6K, S6 and 4EBP1, involved in protein synthesis and survival. By contrast, ATM inhibits mTORC1. We investigated the phosphorylation status of mTOR (Fig. 4A) and its downstream targets at basal conditions and after treatment with H_2O_2 at different time points (Fig. 4B). We found that in lymphoblastoid cell lines from A-T patients, the phosphorylation status of mTOR, S6K, S6 and 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 mTORC1 and of its downstream molecules, as compared to the control.

3.4. Betamethasone pre-treatment promotes autophagy inducing autophagosome formation and degradation

SQSTM1/p62 and LC3 are 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 [29]. Its accumulation reveals an impaired autophagic flux [19]. LC3-II amount correlates with the number of autophagosomes, and is used as indicator of the autophagosome formation [30]. 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 lymphoblastoid cells from A-T patients, at basal conditions, SQSTM1 levels are accumulated as compared to controls (Fig. 5A). Such increase was more evident in

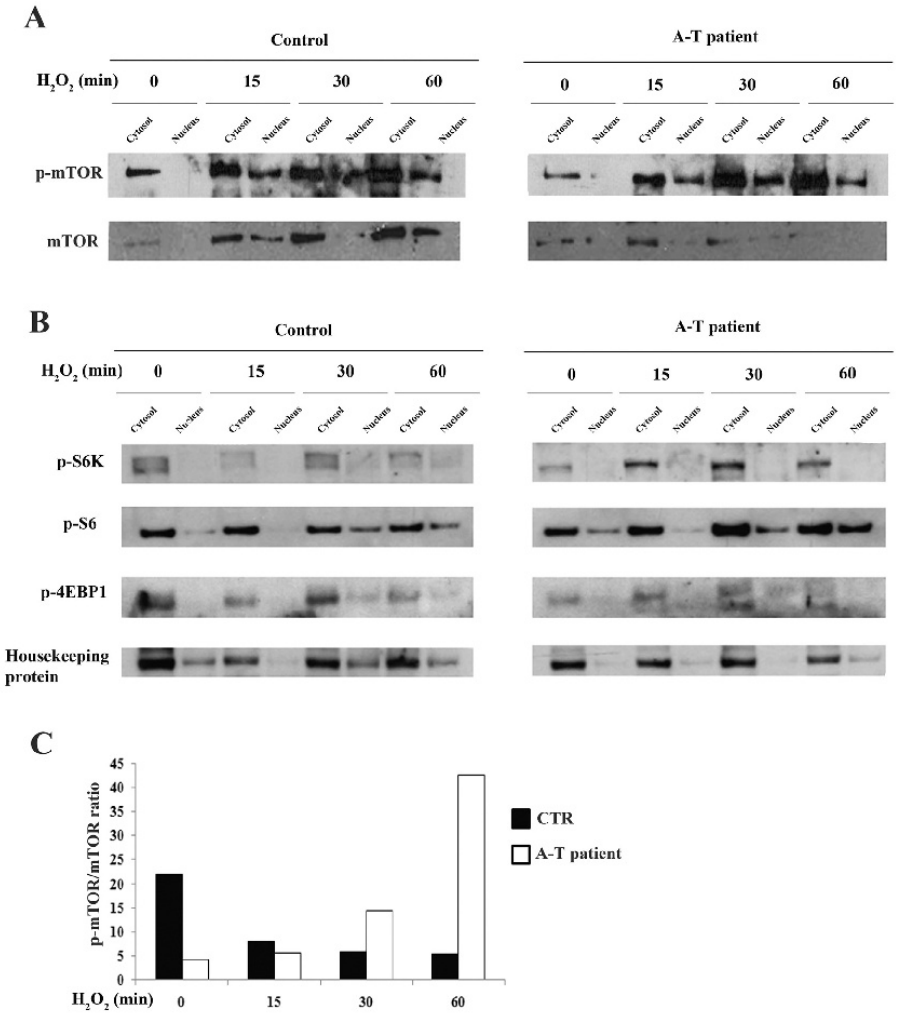

Fig. 4. Autophagy is increased in A-T patients despite an activated mTOR pathway. Lymphoblastoid cells from the A-T patient and control were treated with 0.2 mM of H_2O_2 for different time points. (A) Fractioned lysates were loaded for detection of mTOR and p-mTOR. (B) Fractioned lysates were also loaded on another gel, for detection of p-S6K, p-S6, p-4EBPI and housekeeping protein. (C) p-mTOR/mTOR ratio in lymphoblastoid cells from the A-T patient and control.

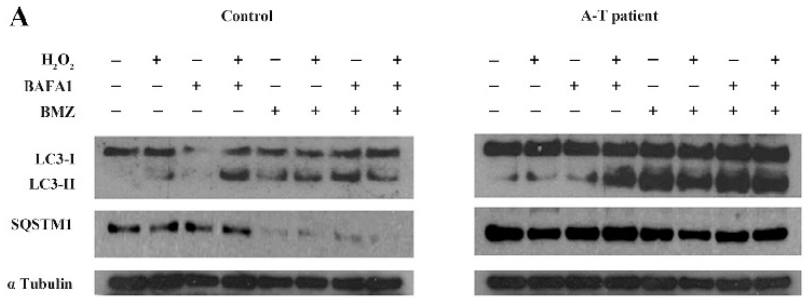

Fig. 5. Betamethasone pre-treatment promotes autophagy inducing autophagosome formation and degradation. (A) Lymphoblastoid cells from the A-T patient and healthy control were pre-treated or not with Betamethasone (BMZ) (80 nM) for 2 h, and H_2O_2 (0.2 mM) for 0.5 h in the presence or absence of 100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

the presence of stress induced for 30′ by H_2O_2 (Fig. 5A). After treating for 1 h with BAFA1, we found that degradation of SQSTM1 was inhibited (Fig. 5A), as expected. Furthermore, a synergic effect of H_2O_2 and BAFA1 treatment resulted in a remarkable increase of SQSTM1 level (Fig. 5A). We also provided evidence that, in lymphoblasts from A-T patients at basal conditions, BMZ pre-treatment is able to promote the degradation of SQSTM1, as shown in Fig. 5A. However, BMZ is ineffective in counteracting the effect on the molecule of the H_2O_2 induced stress (Fig. 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, BAFA1 increased LC3-II expression 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 BMZ positive effect on the modulation of the autophagic process.

Collectively, these results suggest that in A-T patients the process of APs maturation is active, while the fusion process between APs and ALs is inappropriate, the latter being the overall limiting step in the autophagic process. Moreover, we documented a positive effect of Betamethasone pre-treatment on molecules implicated in the cell clearance apparatus and promotion of autophagosome formation and subsequent degradation.

4. Discussion

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 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 cells from altered intracellular components [31].

Autophagy is a dynamic flux including vesicle biogenesis, fusion between APs and lysosomes and, eventually, content 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. [32] 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.

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 [33]. Among the genes involved in lysosome functionality, cathepsins, 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 [21]. 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 that observed in A-T lymphocytes. CST E is an intracellular aspartic proteinase that is predominantly distributed in immune-related cells. The lack of CST E induces several abnormal membrane trafficking events resulting in the impairment of autophagic flux and the accumulation of toxic proteins and/or damaged organelles [34]. CST A is a serine protease, which regulates the lysosomal activity and stability of several lysosomal enzymes such as glycosidases, beta-D-galactosidase, as well as the transport of neuraminidase to mature lysosomes. CST A also triggers the degradation of LAMP 2A receptor involved in the autophagic process [35]. Similarly, in macrophages, the absence of CST S promotes an accumulation of APs [36], 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 hydrolase 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 [24]. The subcellular redistribution 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 homolog 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 AP 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 [25,37]. Both Vps11 and Vps18 are subunits of the Vps-C core complex also composed of Vps16 and Vps33 [38]. In eukaryotic organisms, the Vps-C complex acts as a 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 [38]. Additional data demonstrate that Vps18 plays a prominent role in the process [39]. 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 by an altered regulatory control of the process. The increase in kinesins expression may be interpreted as an attempt to overcome the inappropriate AP-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.

Besides the nuclear activity of ATM, there is evidence that the molecule is also localized in the cytosol, within synaptosomal fractions [40], and, in particular, on the peroxisomes [41], where it induces pexophagy in response to ROS [42]. It is also well documented that ATM, in the cytosol, has a role in the autophagy pathway [12]. In fact, in the presence of elevated ROS levels, ATM activates the tumor suppressor TSC2, through the LKB1/AMPK pathway, to inhibit mTORC1 and, in turn, induces autophagy [43]. In this study, we documented in lymphoblasts from A-T patients, an increase of the phosphorylated S6K, S6 and 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 AP 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.

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 [44,45], 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 lymphoblastoid cells obtained from A-T patients. 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 cells from A-T patients, characterized by an accumulation of un-degraded APs. The molecular studies led us to identify the fusion process between APs and lysosomes the limiting step in the overall process. To address the issue of whether the alterations observed in lymphocytes from A-T patients could also be responsible for neurodegeneration, further studies will be planned in $ATM^{-/-}$ neuronal cell lines.

Abbreviations

A-T	Ataxia Teleangiectasia
AP	Autophagosome
AL	Autolysosome
LC3	Microtubule-associated protein-light chain 3
SQSTM1	Sequestosome 1
BMZ	Betamethasone

Conflicts of interest

The authors declare no financial or commercial conflict of interest.

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CHAPTER IV

“Immunodeficiencies and Autoimmunity”

Even though Immunodeficiencies and autoimmunity may be considered two opposite conditions, deriving from different alterations of the immune system, several evidences suggested that PIDs are often associated with different autoimmune manifestations (95).

Autoimmunity in PIDs may be caused by different mechanisms, including defects of tolerance to self-antigens and persistent stimulation as a result of the inability to eradicate antigens.

This general immune dysregulation leads to compensatory and exaggerated chronic inflammatory responses that lead to tissue damage and autoimmunity.

Each PID may be characterized by distinct, peculiar autoimmune manifestations (96).

In the review published on *Frontiers in Pediatrics,* the main autoimmune manifestations and the pathogenetic mechanism underlying autoimmunity in a specific PID has are summarized.

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MINI REVIEW
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Unbalanced Immune System: Immunodeficiencies and Autoimmunity

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Increased risk of developing autoimmune manifestations has been identified in different primary immunodeficiencies (PIDs). In such conditions, autoimmunity and immune deficiency represent intertwined phenomena that reflect inadequate immune function. Autoimmunity in PIDs may be caused by different mechanisms, including defects of tolerance to self-antigens and persistent stimulation as a result of the inability to eradicate antigens. This general immune dysregulation leads to compensatory and exaggerated chronic inflammatory responses that lead to tissue damage and autoimmunity. Each PID may be characterized by distinct, peculiar autoimmune manifestations. Moreover, different pathogenetic mechanisms may underlie autoimmunity in PID. In this review, the main autoimmune manifestations observed in different PID, including humoral immunodeficiencies, combined immunodeficiencies, and syndromes with immunodeficiencies, are summarized. When possible, the pathogenetic mechanism underlying autoimmunity in a specific PID has been explained.

Keywords: autoimmunity, immunodeficiency, autoimmune hemolytic anemia, immune thrombocytopenia, systemic lupus erythematous

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INTRODUCTION

Immunodeficiencies and autoimmunity may be considered two opposite conditions, deriving from different alterations of the immune system. However, the evidence that primary immunodeficiencies (PIDs) are often associated with different autoimmune manifestations suggests that they could share common pathogenetic mechanisms, which result in a broad immune dysregulation.

Immune system becomes self-tolerant through two main mechanisms called central and peripheral tolerance. As for T cells, central tolerance takes place within the thymus and is mediated by medullary thymic epithelial cells (mTEC), which express tissue-specific antigens under the control of the the transcription factor autoimmune regulator (AIRE) (1–3). Developing T-cells recognizing self-antigens receive a signal to die via programed cell death and, thereby, are deleted, through negative selection, from the T-cell repertoire (4, 5). As for B cells, negative selection of autoreactive cells takes place within the bone-marrow. Different mechanisms, including immunological ignorance, anergy, and suppression through regulatory T cells (Treg) are implicated in the control of self-reactive cells, which escape central tolerance and reach the periphery. For example, the ligation of T-cell receptor (TCR), in the absence of costimulatory molecules, makes the cells inable to express effector functions like cytokine secretion, leading to anergy (6). The control of the expression of the costimulatory molecules CD80 and CD86 is a major mechanism of peripheral tolerance (6).

In some cases, the inability to eradicate foreign antigens may lead to an exaggerated chronic inflammatory responses and autoimmunity (7–10), through several mechanisms, including

molecular mimicry, by-stander activation, epitope spreading, and cryptic antigens.

Each PID is characterized by distinct, peculiar autoimmune manifestations (**Tables 1** and **2**), but the mechanisms may differ.

In this review, we will describe the main autoimmune manifestations observed in different PIDs, including humoral immunodeficiencies, combined immunodeficiencies, and syndromes with immunodeficiencies, and, when possible, we will try to explain the pathogenetic mechanism underlying autoimmunity in a specific PID.

AUTOIMMUNITY IN HUMORAL IMMUNODEFICIENCIES

Selective IgA Deficiency

Selective IgA deficiency (SIgAD) is the most common PID in humans (11). According to European Society for Immunodeficiencies (ESID) criteria, SIgAD is defined by the presence of serum IgA levels <0.07 g/l in the absence of IgG and IgM deficiencies, after the age of 4 years (12). Patients with SIgAD have an increased risk to develop allergies and autoimmune manifestations, including juvenile idiopathic arthritis, rheumatoid arthritis, thrombocytopenic purpura, hemolytic anemia, inflammatory bowel disease (IBD), Sjogren's disease, polyarteritis nodosa, systemic lupus erythematosus (SLE), celiac disease, and insulin-dependent diabetes mellitus (T1D) (**Table 1**) (13). Little is known about the pathogenesis of SIgAD and the predisposition to autoimmunity in these patients. Specific human leukocyte antigen (HLA) haplotypes, including 8.1, DR7, DQ2, DR1, and DQ5 have been identified in patients with SIgAD at higher risk autoimmune diseases (14), such as SLE, autoimmune thyroiditis, and celiac disease. In a recent study, the identification of single nucleotide polymorphisms in the IF1H1 gene encoding for an interferon inducible RNA helicase 1 protein and of a mutation in the CLEC16A gene in SIgAD patients has been associated with the development of autoimmune manifestations (14). Jacob et al. hypothesized that IgA exerts a protective role against autoimmunity. In particular, the interaction between the Fc fragment of IgA receptor and the immunoreceptor tyrosine-based activation motif deactivates the

TABLE 1 | Autoimmune manifestations in humoral immunodeficiencies.

1. SIgAD
   - Juvenile idiopathic arthritis
   - Rheumatoid arthritis
   - ITP, AHA
   - IBD
   - Sjogren's disease
   - Polyarteritis nodosa
   - SLE
   - Celiac disease
   - T1D
2. CVID
   - ITP, AHA
   - SLE
   - IBD
3. PRKCD deficiency
   - Glomerulonephritis
   - Polychondritis
   - Antiphospholipid syndrome
   - LES
4. LRBA deficiency
   - IBD
   - AHA, ITP
   - Granulomatous-lymphocytic interstitial lung disease
   - T1D
   - Neutropenia
   - Chronic autoimmune hepatitis
   - Eczema
   - Uveitis
   - Alopecia
5. Hyper-IgM syndrome
   - IBD
   - Seronegative arthritis
   - Hypothyroidism
   - SLE
   - Autoimmune hepatitis
   - ITP, AHA
   - T1D
   - Uveitis

SIgAD, selective IgA deficiency; ITP, immune thrombocytopenia, AHA, autoimmune hemolytic anemia; IBD, inflammatory bowel disease; SLE, systemic lupus eritematous; T1D, type 1 diabetes mellitus; CVID, common variable immunodeficiency.

TABLE 2 | Autoimmune manifestations in combined immunodeficiencies and in syndromes with immunodeficiency.

1. RAG-1/2 deficiency RMRP, ADA, IL2RG, Artemis, DNA ligase IV, ZAP70, and IL7Ra deficiency
   - Omenn syndrome (erythrodermia, alopecia, hepatosplenomegaly, and lymphoadenopathy)
2. PNP-deficiency, and mutations of ADA, DNA ligase IV, Cernunnos, ORAI1 and STIM1, and hypomorphic RAG1 mutations
   - AHA, ITP
3. Wiskott–Aldrich
   - AHA
   - Autoimmune neutropenia
   - Vasculitis
   - IgA nephropathy
   - Polyarthritis
   - IBD
4. DiGeorge syndrome
   - ITP, AHA
   - Autoimmune arthritis
   - Autoimmune hepatitis
   - Vitiligo
   - IDB
   - Autoimmune endocrinopathy
5. Ataxia telangiectasia
   - Psoriasis
   - Autoimmune thyroid disease
6. STAT1 gain of function
   - Autoimmune thyroid disease
   - IPEX-like phenotype (eczema, enteropathy, T1D, hypothyroidism, and growth hormone insufficiency)
7. STAT3 gain of function
   - Early onset autoimmunity (neonatal diabetes, enteropathy, desquamative interstitial pneumonitis, and posterior uveitis)

ITP, immune thrombocytopenia; AHA, autoimmune hemolytic anemia; IBD, inflammatory bowel disease.

pathways of immune response carrying this motif through a partial phosphorylation (15). Moreover, the evidence of antibodies to bovine milk proteins in over 60% of IgA deficient patients may help explaining the association between SIgAD and inflammatory diseases of gastrointestinal tract (16, 17).

Common Variable Immunodeficiency

Common Variable immunodeficiency (CVID) is a heterogeneous group of disorders characterized by a primary antibody deficiency, usually manifesting between the second and fourth decades of life with a mean age at onset of 26.3 years (18). It is the second most common immunodeficiency with an estimated prevalence ranging from 0.073 to 0.977 living patients per 100,000 inhabitants (19). According to the ESID diagnostic criteria, CVID should be taken into account in presence of a marked decrease of IgG and IgA with or without low IgM levels (measured at least twice; <2SD of the normal levels for their age) (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). Moreover, all of the following criteria should be fullfilled: poor antibody response to vaccines (and/or absent isohemagglutinins) or low switched memory B cells (<70% of age-related normal value); secondary causes of hypogammaglobulinaemia have been excluded diagnosis is established after the 4th year of life; no evidence of profound T-cell deficiency (http://esid.org/Working-Parties/Registry/Diagnosis-criteria). More than 25% of CVID patients develop autoimmune complications (18, 20). Other medical conditions may include gastrointestinal infectious or inflammatory disease, lymphadenopathy, splenomegaly, and hematological malignancies (21). Cytopenia is the most common manifestation. Immune thrombocytopenia (ITP) has been found in up to 14% of patients and autoimmune hemolytic anemia (AHA) in up to 7% (22). In most cases (about 60%), the cytopenia precedes the identification of hypogammaglobulinemia (23). SLE has been reported in some rare CVID patient (24), predominantly females (89%). In about 50% of patients, CVID developed within 5 years of the diagnosis of SLE (24). Some patients experience an improvement in SLE symptoms when hypogammaglobulinemia appears (24). Hypogammaglobulinemia can develop because of the use of immunosuppressive treatment (i.e., corticosteroids or immunosuppressants). Unlike CVID, the cessation of therapy should solve hypogammaglobulinaemia. Nevertheless, in some occasion, the duration of post-cessation hypogammaglobulinaemia can be very prolonged, making difficult to understand its origin (25). IBD has been reported in 6–10% of CVID patients (**Table 1**) (22). Many different alterations could help explain the predisposition to autoimmune manifestations. In a subgroup of CVID patients, IL-7 levels were found to be increased (26, 27). IL-7 plays a key role in the expansion of autoreactive T-cell clones in the lymphopenic host (26, 27). Moreover, reduced levels of switched memory B cells and increased levels of activated CD21-low B cells have been associated with autoimmune manifestations in CVID. Increased levels of CD21-low B cells have been identified in SLE, rheumatoid arthritis, and cryoglobulinemia, suggesting a role for these cells in the pathogenesis of autoimmunity (28–30). Most CVID patients present with elevated BAFF levels (27). Of note, increased BAFF levels sustain the expansion of CD21-low B cells in CVID (31). Moreover, studies show that overexpression of BAFF in mice leads to B-cell hyperplasia, splenomegaly, and autoimmunity (32, 33). Different genetic mutations, including TACI, ICOS, BAFF-R, CD20, and CD21 have been associated with increased risk of developing CVID (34–40). Among these genetic alterations, autoimmunity is most common in TACI alterations [18/50 (36%) vs. 112/490 (23%) in wt TACI CVID], in particular, heterozygous C104R mutations (11/20 patients, 55%) (41).

PRKCD Deficiency

A CVID-like disorder associated with multiple features of immune dysregulation, including glomerulonephritis, lymphadenopathy, relapsing polychondritis, and antiphospholipid syndrome has been recently described in a 12-year-old patient born to consanguineous parents of Turkish origin (42). Genetic studies revealed a mutation of *PRKCD* gene, leading to a complete absence of the protein. PRKCD deficiency has also been reported in three siblings with LES (**Table 1**) (43). PRKCD plays a key role in the regulation of cell survival, proliferation, and apoptosis (44). PRKCD deficiency in mice seems to be related to a defective deletion of autoreactive B cells during B-cell development, due to impaired proapoptotic extracellular signal-regulated kinase signaling (45, 46).

LRBA Deficiency

LPS-responsive beige-like anchor protein (LRBA) deficiency is a novel PID caused by either homozygous or compound heterozygous mutations in *LRBA* that abolish LRBA protein expression. This PID is characterized by early onset hypogammaglobulinemia, autoimmune manifestations, susceptibility to IBD, and recurrent infections (47). However, it has been also described in patients with IBD with or without antibody deficiency (48, 49), in patients with autoimmune manifestations without hypogammaglobulinemia (50), or in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX)-like disorder (51). The main clinical manifestations of LRBA deficiency are immune dysregulation (95%), followed by organomegaly (86%) and recurrent infections (71%). The most common autoimmune manifestations are enteropathy (59%), AHA (50%), and ITP (50%). A lower number of patients presented granulomatous-lymphocytic interstitial lung disease (36%), T1D or neutropenia (22%), chronic autoimmune hepatitis (13%), eczema and uveitis (9%), and alopecia (4.5%) (**Table 1**). LRBA is a highly conserved multidomain protein implicated in regulating endosomal trafficking, cell proliferation, and survival. LRBA deficiency is associated with increased apoptosis and altered phenotype of Treg cells, which express lower levels of key effector proteins involved in Treg cell suppression, such as CD25 and CTLA-4. This results in decreased frequency, aberrant phenotype, and decreased suppressive function of such cells. These alterations might play a critical role in the ubiquitous autoimmune manifestations of the disease.

CTLA-4 Haploinsufficiency

CTLA-4 haploinsufficiency has been recently associated with lymphoproliferation, lymphocytic infiltration, autoimmunity,

peripheral B-cell lymphopenia, hypogammaglobulinemia, and increased CD21lo B cells (52). In mouse models, homozygous CTLA-4 deficiency leads to a lethal autoimmune phenotype characterized by multiorgan lymphocytic infiltration and destruction (53, 54) resambling FOXP3 deficiency (55–57). CTLA-4 plays a key role in immune tolerance. Recent studies show that CTLA-4 is able to suppress the expression of CD80 and CD86 from antigen presenting cells (APCs) via transendocytosis (58). The depletion of the costimulatory ligands reduces T cell activation (59).

Activated Phosphoinositide 3-Kinase δ Syndrome

Activated phosphoinositide 3-kinase δ syndrome (APDS) 1 and 2 are PID resulting from autosomal dominant mutations in PI3KCD and PIK3R1, respectively (60, 61). Autoimmune manifestations are reported in 34% of APDS1 patients. The clinical manifestations included cytopenias (AHA or tri-lineage cytopenia), glomerulonephritis, exocrine pancreatic insufficiency, autoimmune thyroid disease, seronegative arthritis, recurrent pericarditis, sclerosing cholangitis, and gastrointestinal nodular mucosal lymphoid hyperplasia (61). Autoimmune manifestations have been reported in the 17% of the APDS2 patients. They included ITP, AHA, Evans syndrome, T1D, chronic arthritis, autoimmune hepatitis, and chronic eczema (60). PI3Kδ is implicated in the regulation of Treg cell function. Studies suggest that PI3K is an important target for the treatment of different autoimmune conditions.

Hyper-IgM Syndrome

Hyper-IgM syndrome (HIGM) is a group of disorders characterized by alterations of immunoglobulin receptor isotype switching, leading to normal or elevated IgM antibody and very low IgA, IgG, and IgE antibodies (62). Alterations in different genes implicated in CD40-CD40L pathway involved in B cell activation, class switch recombination or somatic hypermutation have been identified in HIGM. Seven different forms of HIGM have been till now described. Most of the cases (65–70%) are due to mutations of the gene encoding for CD40 ligand (CD40L) on the X chromosome, leading to HIGM1 (63). The other forms are due to mutations of AID (HIGM2), CD40 (HIGM3), UNG (HIGM5), NEMO (HIGM6), and IkBα (HIGM7). No genetic defect has been so far identified for HIGM4.

Autoimmunity has been described in all forms of HIGM. HIGM1 patients have an increased risk to develop IBD, seronegative arthritis, hypothyroidism, and SLE (64). In the 21% of patients affected with HIGM2 autoimmune hepatitis, ITP, T1D, IDB, and uveitis have been described (65). In addition, in patients with NEMO defects, AHA, IBD, and arthritis have been described (**Table 1**) (66). Studies on transgenic mouse models suggest that CD40–CD40L interactions is involved in the elimination of autoreactive B cells (67). In fact, an increase of circulating polyreactive B cells and a significant decrease of CD25+Foxp3+Treg cells have been reported in CD40L-deficient patients suggesting defects of the peripheral B-cell tolerance mechanism. An imbalanced production of cytokines, including as IL-1, IL-8, IL-6, IL-10, IL-12, and tumor necrosis factor (TNF)-α may be observed in CD40-deficient patients (68). This impairment is the consequence of the involvement of CD40–CD40L interaction in T-cell dependent macrophage-mediated immune response, implicated in the maturation of dendritic cells and regulation of the T-cell activation. The transcription factor NFkB plays a key role in the regulation of pro-inflammatory responses. Recent studies suggest that gut epithelial cells are directly implicated in the control of epithelial integrity and the regulation of the interaction between the mucosal immune system and gut microflora. In mice, NEMO deficiency causes a severe chronic intestinal inflammation, which has been associated with apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides, and translocation of bacteria into the mucosa. The chronic inflammatory response observed within the colon, is dominated by innate immune cells, as suggested by the upregulation of IL1b, IL6, TNF, and Ccl2 and by the infiltration of large numbers of dendritic cells and granulocytes in the colon. Eventually, also T lymphocytes are involved, as suggested by the presence of lymphoid follicles and a massive infiltration with CD4+ T cells in the gut mucosa.

COMBINED IMMUNODEFICIECIES

Severe combined immunodeficiency (SCID) is a group of different PIDs characterized by a severe deficiency of the cellular and humoral immune system. SCID phenotype may be due to a variety of different mutations. From a clinical point of view, SCID is characterized by recurrent severe infections, chronic diarrhea, and failure to thrive (69, 70). The clinical presentation may drive the diagnosis toward a specific molecular cause of SCID (69). Patients affected with SCID often develop autoimmune manifestations. This may appear surprising in that SCID patients, who are unable to mount any immune response to foreign pathogens, may paradoxically develop autoimmune phenomena. Alterations in both central and peripheral tolerance have been described in SCID patients (71).

Autoimmunity in Omenn Syndrome

Omenn syndrome (OS) is a SCID inherited in an autosomal recessive manner, caused by homozygous or compound heterozygous mutations in recombinase activating gene 1 (RAG1) or RAG2, implicated in V(D)J recombination, which represents a crucial step in T- and B-cell differentiation. OS has also been associated with hypomorphic mutations in other different genes, including RMRP, ADA, IL2RG, Artemis, DNA ligase IV, ZAP70, and IL-7Ra deficiency (72, 73). Signs of OS, including oligoclonal T-cell expansion, generalized rash, and lymphadenopathy have been reported in some patient affected with DiGeorge syndrome. This rare condition is known as atypical complete DiGeorge syndrome (74). Apart from recurrent infections, patients affected with OS also show features of autoimmunity, including erythrodermia, alopecia, hepatosplenomegaly, and lymphadenopathy (**Table 2**). The hallmark of the syndrome is the expansion and activation of a peripheral oligoclonal population of autoreactive T cells, due to defective central (75, 76) and peripheral tolerance mechanisms (77). Studies suggest

that in OS, defective AIRE expression may lead to inadequate expression of tissue-specific self-antigens by mTEC, impairing central tolerance. In these patients, the T-cell compartment is composed by a high proportion of autoreactive T cells, which are able to expand in peripheral tissues leading to the clinical symptoms. Similarly, alterations in central tolerance may be implicated in the pathogenesis of immune manifestations also in PIDs characterized by ineffective thymopoiesis, such as the DiGeorge syndrome or in SCID characterized by partial defects of the T-cell maturation, such as IL-7alfa, common γ chain, or ARTEMIS defects. The persistent infectious/inflammatory state and the presence of immunologic "space," which increases the ability of T cells to respond to an excess of cytokines or antigens, impairs peripheral tolerance in SCID patients. Treg population may be also affected in SCID patients.

Autoimmune Manifestations in SCID Due to IL7R Mutations

IL7Ra deficiency is responsible of the majority of T-B+NK+ cases (72) characterized by an increased susceptibility to severe and opportunistic infections. In a few cases, autoimmune manifestations have been reported (72, 73). Autoimmune manifestations presented with OS in one infant (73), and cytopenias in three other cases. Autoimmune cytopenias have been also described in some patients with PNP-deficiency, and mutations of ADA, DNA ligase IV, Cernunnos, and hypomorphic RAG1 mutations (**Table 2**) (69, 78–82).

Ca++ Channellopathies Due to Mutations in ORAI1 and STIM1

Null or loss-of-function mutations in ORAI1 or STIM1 are associated with a SCID-like disease characterized by recurrent and chronic infections, autoimmunity, ectodermal dysplasia, and muscular hypotonia in the presence of numerically intact T, B, and NK cells. Symptoms usually manifest in the first year of life. Lymphoproliferation, AHA, and ITP are very common in patients with STIM1 mutations (**Table 2**). Autoimmunity may derive from alterations of negative selection of autoreactive T cells and/or B cells during their development. In fact, Ca2+ signals are implicated in TCR and BCR signaling and thus potentially influence the selection thresholds in immature T and B cells. Moreover, a reduced frequency of Treg cells has been observed in STIM1-deficient patients (83, 84) and in one patient with ORAI1 p.R91W mutation.

SYNDROMES WITH IMMUNODEFICIENCY

Wiskott–Aldrich

Wiskott–Aldrich syndrome is a very rare immunodeficiency, characterized by thrombocytopenia, eczema, and recurrent bacterial infections appearing in the first months of life. Other features includes humoral and cellular immunodeficiency, defects of the innate immunity (85–87), increased risk to develop autoimmune manifestation and malignancies, impaired apoptosis (88, 89), and defective cell motility (90). The gene responsible for WAS (WASP) is located on the X chromosome and encode for WASP protein, which is only expressed in the cytoplasm of hematopoietic cells. WASP protein plays a major role in the transduction of the signals from the cell surface to the actin cytoskeleton, which regulates actin polymerizationand the formation of actin filament (91, 92). WAS patients are at a higher risk of developing autoimmunity and most of WAS patients (about 40%) are affected by at least one autoimmune manifestation (93, 94). The most common autoimmune manifestations include AHA, autoimmune neutropenia, vasculitis, and IgA nephropathy with or without the association with Henoch–Schönlein purpura, polyarthritis and IBD (**Table 2**) (87, 93–95). Studies suggest that a defect in Treg cells could be implicated in the pathogenesis of autoimmune manifestations (96, 97). In fact, Treg cells, isolated from WAS patients, show a reduced ability to suppress effector T-cell proliferation and IFN-γ production (98, 99). On the contrary, Treg cell development is not impaired in these patients. In addition, Treg cells from WASp−/− mouse show a reduced granzyme B secretion, which results in the inability to suppress B-cell proliferation and apoptosis. Furthermore, studies on mouse models show that Treg cells from WASp−/−mouse are not able to prevent the development of autoimmunity in scurfy mice (Foxp3-deficient) (98–100). Also B cells may be implicated in the pathogenesis of autoimmune manifestations in WAS patients. Studies show that selective deletion of WASP in B cells leads to the production of autoantibodies and the development of autoimmunity (101, 102).

DiGeorge Syndrome

Autoimmune manifestations have been reported in about the 10% of patients with DiGeorge syndrome (103–105). Autoimmune disorders include mainly autoimmune cytopenias (ITP, AHA) (106–108), autoimmune arthritis (107), autoimmune hepatitis, vitiligo, IDB, and autoimmune endocrinopathy (**Table 2**) (109). Impaired T-cell development in an abnormal thymus may result in altered central tolerance and escape of self-reactive T. Thymic abnormality may also result in impaired generation of Treg (96, 110, 111).

Ataxia Telangiectasia

Patients with ataxia telangiectasia (A-T) have increased frequency of autoimmune disorders (112), including psoriasis and autoimmune thyroid disease (**Table 2**). Loss of suppressor T-cell function has been described as responsible for the development of autoimmune disease.

STAT1 Gain of Function

Increased incidence of autoimmunity has been reported in heterozygous STAT1 gain-of-function (GOF) mutations (113). The main clinical features of the syndrome include chronic mucocutaneous candidiasis (CMC) (114–118), disseminated coccidioidomycosis, and histoplasmosis (116, 119), recurrent sinopulmonary infections and pneumonias (with or without bronchiectasis), herpes virus infections, blood-borne infections, squamous cell cancer, and cerebral aneurysms (116, 120). The most common autoimmune manifestation is thyroiditis, but in

some case patient may show an IPEX-like phenotype (121). Number and function of Treg cells are usually normal and the pathogenesis of IPEX-like disease remains unclear (121).

STAT3 Gain of Function

Recent studies show that activating STAT3 mutations may lead to autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease (122). The autoimmune manifestations are early onset and include neonatal diabetes and some rare disorders, such as desquamative interstitial pneumonitis and posterior uveitis (123). Patients with activating STAT3 mutations show a reduced number of Th17 cells, decreased IL-17 production, and deficiency of Treg, NK, and dendritic cells (123). Autoimmunity may develop as a consequence of the impaired Treg development.

CONCLUSION

Autoimmunity and immunodeficiencies represent two opposite conditions, which may coexist in the context of a general immune dysregulation. Even though different mechanisms have been identified to explain autoimmunity in PIDs, the pathogenesis of autoimmunity remains unexplained in most of the cases. Considering this strong association, underlying immunodifiency should be always excluded in particular in presence of early onset or multiple autoimmune manifestations (124).

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter V

Conclusive Remarks

The immune system is a complex integrated network of chemical and cellular mediators that develops 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-threatening 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 pathogenic 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 under diagnosed 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.

In this thesis, during the three years of my PhD program, I have contributed to elucidate "*Novel insights in the pathogenesis of congenital immunodeficiencies*", through the clinical, cellular, functional and molecular study of some Immunological disorders. In particular, my research work has been focused on the positive effect of oral betamethasone administration on the lymphocytes functionality in patients affected of Ataxia-Telangiectasia, and the identification of the molecular checkpoint responsible for the partial functional rescue in lymphocytes of the patients affected with this disease.

A further project described in this thesis concerns the evaluation of the pathogenic mechanisms, including the evaluation of cellular response to DNA injury, in patients with increased IgM levels, impaired B-cell homeostasis and high incidence of lymphoproliferation. NGS technologies revealed in two of them mutations in the PIK3R1 and ITPKB genes, implicated in T- and B-cell development and survival. This study highlights the possible role of polyclonal hyper IgM as biomarker of immune dysregulation and cancer susceptibility.

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

CURRICULUM VITAE

europass Curriculum vitae

PERSONAL INFORMATION

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EDUCATION AND TRAINING

10/2015–10/2018

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 "Clinical and Experimental Medicine" with a research project focused on the evaluation of the role of IL-7/IL7Ra axis in the pathogenesis of Ataxia Teleangiectasia.

09/2012–09/2014

Bachelor in Biotechnological Sciences (II level degree) at the "Federico II" University of Naples, with a thesis entitled: "Studio dei meccanismi di regolazione dell'autofagia in linfociti ottenuti da pazienti affetti da Atassia Teleangiectasia ". Vote: 110/110 with honors.

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

Internship at Department of Translational Medical Sciences of the "Federico II" University of Naples, Naples, Italy, focused on the on the evaluation of the role of autophagy in the pathogenesis of Ataxia Teleangiectasia

09/2007–12/2010

Bachelor in Biotechnological Sciences (I level degree) at the "Federico II" University of Naples with a thesis entitled: "Caratterizzazione Del Profilo Immunologico Periferico In Pazienti Affetti Da Fibrosi Polmonare Idiopatica". Vote: 110/110

ISCED6

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

Internship at the Department of Immunology of the Monaldi Hospital of Naples, Naples, Italy, focused on the characterization of immunological peripheral profile in patients with idiopathic pulmonary fibrosis.

PERSONAL SKILLS

Mother tongue(s) Italiano

Other language(s)

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

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" 2015-2018 (VQR 2015-2018).

ADDITIONAL INFORMATION

Scientific Interests

rests **SCIENTIFIC SOCIETIES**

European Society for Immunodeficiencies (ESID) Junior Member

APPLICATIONS TO GRANT PROPOSALS

Dr. Prencipe gave a contribution in writing the following applications:

1. 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”.
2. 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”.
3. 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”.
4. 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”.
5. Project “Ricerca Finalizzata”, Ministero della Salute, 2016, entitled:
   "Analysis of circulating and tissue-specific micro-RNAs in patients with rare diseases: identification of molecular biomarkers of disease progression and therapeutic efficacy”.

SCIENTIFIC PRODUCTION

LIST OF PUBLICATIONS:

- Prencipe MR, Cirillo E, Giardino G, Gallo V, Menotta M, Magnani M, Scalia G, Barone MV, Del Vecchio L, Pignata C. In Ataxia-Telangiectasia, Betamethasone modulates IL-7/IL-7Rα axis by interfering on intracellular trafficking. Frontiers in Immunology 2018 (submitted).

- Gallo V, Cirillo E, Sannino A, Giardino G, Prencipe MR, Del Vecchio L, Scalia G, Martinelli V, Di Matteo G, Saunders CJ, Durandy AH, Moschese V, Scarfì MR and Pignata C. Elevated polyclonal IgM levels, impaired B cell homeostasis and increased susceptibility to lymphoproliferation. Journal of Clinical Immunology 2018 (submitted).

- D'Assante R., Fusco A., Palamaro L., Polishchuk E., Polishchuk R., Grieco V., Prencipe M.R., Ballabio A., Pignata C. Abnormal cell-clearance and accumulation of autophagic vesicles in lymphocytes from patients affected with Ataxia-Teleangiectasia. Clin Immunol. 2017; 175:16-25.

- Giardino G, Gallo V, Prencipe R, Gaudino G, Romano R, De Cataldis M, Lorello P, Palamaro L, Di Giacomo C, Capalbo D, Cirillo E, D'Assante R, Pignata C. Unbalanced Immune System: Immunodeficiencies and Autoimmunity. Front Pediatr. 2016; 4:107.

MEETING ABSTRACTS AND COMMUNICATIONS:

- **Prencipe MR**, Cirillo E, Giardino G, Gallo V, Palamaro L, Scalia G, Del Vecchio L, Pignata C. In Ataxia-Telangiectasia, oral Betamethasone administration ameliorate lymphocytes functionality through modulation of the IL-7/IL-7Rα axis. Ataxia Telangiectasia Clinical Research Conference- Naples, Italy 29 November – 1 December 2018.
- **Prencipe MR**, Cirillo E, Giardino G, Gallo V, Palamaro L, Scalia G, Del Vecchio L, Pignata C. In ataxia-telangiectasia, oral betamethasone administration interferes on IL-7/IL-7Rα axis in lymphocytes functionality through modulation of intracellular trafficking. ESID 2018 Biennal Meeting, Lisbon, Portugal.
- **Prencipe MR**, D'Assante R., Cirillo E, Palamaro L, Giardino G, Gallo V, Scalia G, Del Vecchio L, Pignata C. The potential role of Betamethasone in modifying lymphocytes functionality through modulation of intracellular trafficking and in particular of IL-7Rα. Ataxia-telangiectasia Workshop (ATW) March 20-24, 2017 | IFOM, Milan, Italy
- Giardino G, Gallo V, Cirillo E, **Prencipe MR**, Di Giacomo C, Palamaro L, D'Assante R, Lougaris V, Saunders CJ, Pignata C. Late onset combined immunodeficiency of unknown genetic origin presenting with persistent EBV-viremia, colitis and pulmonary aspergillosis. ESID 2016 Biennal Meeting, Barcelona, Spain.
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AWARDS

- Best e-poster with the abstract "Altered fusion process between autophagosomes and lysosomes in lymphocytes from patients affected with Ataxia Telangiectasia (A-T)" at the 17th Biennal Meeting of the European Society of Primary Immunodeficiency (ESID), Barcellona, September 2016.

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