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ROLE OF MUNC13-4, SAP AND IL-17 IN THE DEVELOPMENT OF AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

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1. PREFACE

During my period as a PhD student, I worked on projectsmainly focused on genetic variations and hematologic alterations associated with the development of autoimmune lymphoproliferative syndrome (ALPS). ALPS is an autoimmune disease due to defective lymphocyte apoptosis resulting in accumulation of polyclonal lymphocytes in the lymph nodes and the spleen, and expansion of T cells expressing the T cell receptor αβ (TCRαβ) but lacking the CD4 and CD8 coreceptors, and therefore named double-negative (DN) T cells; patients often display autoimmune manifestations, mainly hemocytopenias.The disease is caused by genetic mutations that decrease the function of the Fas death receptor and that display an autosomal dominant inheritance with incomplete penetrance. Most patients carry a heterozygous mutation in the *FAS* gene (ALPS-FAS), whereas few patients carry mutations in the *FAS LIGAND* (ALPS-FASLG) or *CASPASE10* (ALPS-CASP10) genes. Moreover, a substantial number of patients carry somatic mutations in *FAS* in the DN T cell population (ALPS-sFAS), whereas the causal mutation is unknown in another substantial proportion of patients (ALPS-UND). Our laboratoryalso described patients with lymphadenomegaly/splenomegaly, autoimmune manifestations and defective Fas function, but lacking expansion of DN T cells. This disease has been named Dianzani Autoimmune Lymphoproliferative Disease (DALD) (OMIM reference #605233), and several features indicate that it may have a genetic component in involving the Fas pathway. Nevertheless, in both ALPS and DALD, several evidences suggest that other gene alterations, apart fromthose of *FAS*, *FASLG* or *CASPASE10*,may influence the disease development as disease modifier gene. Our laboratory, indeed,demonstrated that polymorphic variations of the *OSTEOPONTIN* (*OPN*) and *PERFORIN*(*PRF-1*) genes may be susceptibility factors for ALPS and DALD development and may influence disease expression. Osteopontin is a proinflammatory cytokine capable to inhibit lymphocyte apoptosis, whereas perforin is involved in cell-mediated cytotoxicity of Natural Killer (NK) cells and Cytotoxic T Lymphocytes (CTL), involved in the anti-viral response but also in the immune response homeostasis.

The research described in this thesis stemmed from these works and analyzed the role played in ALPS and DALD by

1. Variations of the*UNC13D* gene coding for Munc13-4involved in perforin function(Boggio E, et al. *PLoS One*. 2013).

2. Variation of the*SAP* gene (SLAM-Associated Protein)involved in NK function (Boggio E, et al. *Human Immunology*. 2012).
3. Interleukin (IL)-17, a proinflammatory cytokine that may work in tandem with osteopontin (Boggio E, et al.*Blood.*2013*)*. In this work, I shared the first authorship with E.Boggio.

2. INTRODUCTION

2.1 Immune defense

The immune response proceeds through an early inflammatory response recruiting relatively non-specific effector mechanisms that are immediately ready to use, and a lateadaptive response recruiting highly specific effector mechanisms requiring several days to be activated.Key cells of inflammation are granulocytes and macrophages, which eliminate the infectious agents primarily through phagocytosis and cooperate with humoral factors including the complement system and the metalloproteinase network.Key cells of the adaptive immune response are lymphocytes including T and B lymphocytes, which specifically recognizemolecules of infectious agents through specific antigen receptorswhich are T Cell Receptors (TCR) for T lymphocytes and antibodies for B lymphocytes. The antibodies directly recognize the antigens in their native form and can be secreted in a soluble form. The TCR recognizes the antigen (usually a protein) only after it has been processed and presented on molecules of the major histocompatibility system (MHC) by antigen presenting cells (APC), such as macrophages, dendritic cells or B lymphocytes.T lymphocytes include $CD8^+$CTL killing either virus-infected cells or neoplastic cells, and $CD4^+$ T helper (Th) lymphocytes producing cytokines capable to finely tune the immune and the inflammatory responses.Over 20 years ago, it was first demonstrated that effector Th cells can be categorized into two distinct subsets, Th1 and Th2, based on their cytokine profile. Th1 cells are mainly characterized by production of large amounts of interferon-gamma (IFN-γ) and tumor necrosis factor-beta (TNF-β). Th2 cells mainly secrete interleukin (IL)-4, IL-5, and IL-6. Th1 cells are key players in delayed type hypersensitivity immune response through activation of macrophages andare involved in the host defense against intracellular pathogens.Th2 cells are more efficient in mounting humoralimmune responses, supporting B cell activation,production ofantibodies and eosinophil infiltration. Th2 responsescounteract extracellular pathogens andcounterbalance the Th1 responses.A further Th cell type characterized in the last years includes Th17 producing IL-17 and supporting the inflammation mediated by granulocytes. They seem to have a key role in protecting against extracellular microorganisms due to their ability to secrete the pro-inflammatory cytokines IL-17, IL-21, IL-22, and IL-26, as well as small amounts of IL-6 and TNF-α. Another Th subset comprises regulatory T (Treg) cells that produce IL- 10 and Transforming Growth Factor (TGF)-βand suppress the immune response and inflammation. They include “natural” Treg cells which originate directly from thymic precursors [1], and “induced” Treg cells that can differentiate from several subsets of effector Th cells under the effect of several cytokines [2,3] (Figure 1). Inflammation and the adaptive response are crucial for protection against infectious agents, but can

also cause diseases, such as in autoimmune diseases and allergies, when they are activated in an inappropriate manner [1].

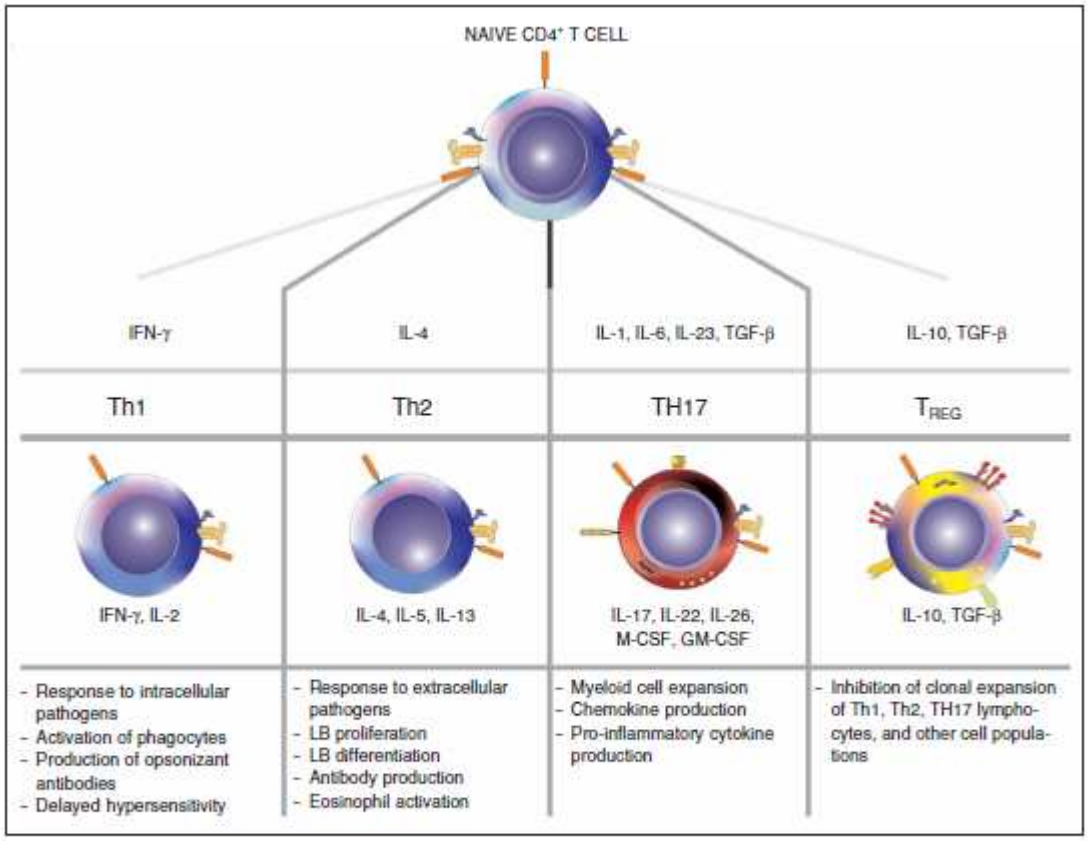

Figure 1. Schematic representation of naive CD4 T cells under different stimuli and possible differentiation pathways. IFN-γ = interferon gamma; IL = interleukin; TGF-β = transforming growth factor beta; Th1, Th2 = T helper cells 1 and 2; T_{REG} = regulatory T cells; M-CSF and GM-CSF = macrophage and granulocyte-macrophage colony-stimulating factor; LB = B lymphocytes.

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2.2 General aspects of autoimmunity

In the early ‘900, Paul Ehrlich realized that some diseases can be caused by an erroneous activation of the immune system against *self*-antigens and termed this condition “horror autotoxicus”. Indeed, it is now well known thata key feature of the adaptive immunity is the ability to discriminate between *self* and *non-self* antigensin order to develop immune responses against the latters and a specific tolerance (*self*-tolerance) against the formers.

A key mechanism of *self*-tolerance is “central tolerance” that occurs during lymphocyte development in the primary lymphoid organs when the precursors of B and T lymphocytes undergo a rigorous process of clonal selection (negative selection) deleting those recognizing *self*-

antigens.In the ‘60s, negative selection was believed to be capable of eliminating all the auto-reactive lymphocytes and thefailure of this processwas believed to be the cause of autoimmune diseases. Inthe late ‘70s,a broad body of experimental evidence showed that the central tolerance mechanisms are not sufficient to eliminate all the auto-reactive lymphocytes, since mature circulating auto-reactive lymphocytes are normally present in healthy subjects. However, the presence of these cells does not inevitably lead to the development of autoimmune diseasessince their activity is controlled by “peripheral tolerance” mechanisms acting by inducing deletion, anergy, and suppression of the auto-reactive lymphocytes.

A key homeostatic mechanism counteracting autoimmunity is the natural tendency of the immune responses to be *self*-limited. When cells of the immune system are activated,they upregulate expression not only of genes involved in proliferation and effector functions, but also of genes sensitizing the activated cell to apoptotic stimuli capable to switch off the immune responseseveral days after its activation. The apoptotic death of “old” activated lymphocytes substantially decreases the risk that they cross-react against *self*-antigens after the elimination of the infectious agent [4].

However, the systems involved in central and peripheral tolerance sometimes may fail, which gives rise to development of autoimmune diseases. This failure may be caused by multiple factors, including infections, environmental factors, and the genetic background, which may inhibit the homeostatic tolerance mechanisms and support the autoimmune effector cells.

Infectionsmay trigger the disease through “antigen mimicry”, in which the immune response against the infectious agenttriggersa crossreactive autoimmune response against *self*-antigens structurally similar to those of the pathogen. A second mechanism is the release of “sequestered” *self*-antigens from tissues damaged by the infection; these *self*-antigens may be reccognized as *non self* because they had not been previously accessible to the immune system and did not activate the tolerance mechanisms. A third mechanism is the “adjuvant effect”exerted by the inflammatoryresponse to the infection; by inducing secretion of high amounts of cytokines and expression of costimulatory molecules,it may increase the responsiveness not only to the infectious agent but also to the *self*-antigens expressed in the inflamed tissue. These mechanisms are not non mutually exclusive and may act together in chronic autoimmune disease to cause the “epitope spreading” of the autoimmune response, i.e. the progressive expansion of the autoimmune response against multiple auto-antigens during the disease progression [5].

A key genetic risk factor for most autoimmune diseases is the gender since many autoimmune diseases (such as systemic lupus erythematosus, myasthenia gravis, scleroderma, multiple sclerosis, and Sjögren's syndrome) are more frequent in females than in males. A key role in this different susceptibility is probably played by sexual hormones, whose receptors are expressed by immune

cells and profoundly influence the immune response in males and females and during the pregnancy. However, this is not the only explanation of female predisposition to develop autoimmunity since recent experiments in mice showed that an independent effect may be directly mediated by genetic factors located in the sex chromosomes.

The gender is not the only genetic factor influencing susceptibility to autoimmune diseases since studies of recurrence risk in families and twins suggest a complex mode of inheritance involving interactions of different combinations of loci influencing the immune response. Despite several whole genome surveys, potent loci have not been detected for most autoimmune diseases. It is likely that most susceptibility genes have a small effect, with the possible exception of the MHC locus, with a high degree of genetic heterogeneity in different individuals. Susceptibility genes may include genes coding for molecules involved in immune response control and immune effector functions. Further genes may be those involved in the immune response switching off, which leads to the homeostatic control of the size of the peripheral lymphocyte pool and reduces the risk of autoimmunity due to cross-reactions between *non-self* and *self* antigens. Intriguingly, some of these genes are also involved in clearance of viral infections. However, even if many studies detected a statistical association of autoimmune diseases with particular alleles of specific genes, often it is not clear whether these alleles are directly involved in the disease development or the statistical association is due to “linkage disequilibrium”, i.e. the physical association of that allele with the allele of a nearby gene which is the real predisposing factor. Moreover, many alleles have been found to be associated in some populations but not in other, which may be explained with the genetic heterogeneity of the populations.

Autoimmune diseases affect 5-10% of the population in Western countries. From a clinical point of view, autoimmune diseases can be divided in“organ-specific” and “systemic”. In organ-specific autoimmune diseases, the immune response is directed to a target antigen selectively expressed by a specific organ, so that clinical manifestations are largely limited to that organ. In systemic autoimmune diseases, the response is directed toward target antigens expressed ubiquitously and therefore involves a broad range of organs and tissues, which are all damaged. Examples of organ-specific autoimmune diseases are Hashimoto's thyroiditis and Graves' disease, affecting the thyroid gland, type I insulin-dependent diabetes mellitus, which affects the pancreatic islets, and multiple sclerosis, affecting the central nervous system. Examples of systemic autoimmune disease are systemic lupus erythematosus and primary Sjögren's syndrome, in which tissues as diverse as the skin, kidneys, and brain may all be affected [5].

From an immuno-pathogenic point of view, autoimmune diseases can be mediated by antibodies or T cells. The first include diseases such as autoimmune emocytopenias, caused by autoantibodies

against various types of blood cells, or systemic lupus erythematosus, caused by the deposition in various tissues of immune complexes formed by antibodies against several *self* antigens, including nuclear antigens such as histones and DNA.This category also includes organ-specific autoimmune disease caused by autoantibodies exerting agonist or antagonist activity on the target organ; for instance, agonist anti-TSH receptor antibodies cause thyroid hyperfunction in Graves-Basedow disease, while antagonist antibodies against the acetylcholine receptor inhibit neuromuscular pulse transmission in myasthenia gravis.

T cell-mediated autoimmune diseases are a growing category and are primarily mediated by Th1, Th17 cells and CTL. Examples of this type of autoimmune diseases are type 1 diabetes mellitus and multiple sclerosis in which autoreactive T lymphocytes attack pancreatic β cells and central nervous system myelin respectively. In these diseases, production of autoantibodies is also observed [6,7]; these are often regarded as useful markers of disease evolution as a consequence of “epitope spreading”, but they are believed to have a modest pathogenic role.

2.3Autoimmune Lymphoproliferative Syndrome (ALPS)

Autoimmune Lymphoproliferative Syndrome (ALPS - OMIM # 601859) is an autoimmune disease of childhood caused by a genetic disorder of lymphocyte apoptosis.In most ALPS patients, the disease is ascribed to mutations of the gene *FAS* coding for the Fas death receptor. Most of them harbor heterozygous germline mutations inherited in an autosomal dominant manner [8,9], butsomatic *FAS* mutations are the second most common cause of the disease [10,11]. In addition, mutations in the genes coding for FAS ligand (*FASL)* and caspase 10 (*CASP10)* have been identified in a minority of patients with ALPS, whereas approximately one-third of patients have yet unidentified genetic defects. Moreover, mutations in the genes encoding for caspase 8 (*CASP8*) and neuroblastoma RAS (*NRAS*) have been detected in patients with ALPS-like diseases [12-17].

These mutations result in the accumulation of proliferating lymphocytes causing chronic lymphadenopathy, splenomegaly, and peripheral expansion of TCR$\alpha\beta^{+}$ CD4/CD8 double-negative (DN) T cells. Patients often develop multilineage cytopenias secondary to sequestration and autoimmune destruction, and display increased risk of B-cell lymphoma development in the adulthood [18-21].

The only pharmacological treatment for ALPS patients is administration of corticosteroids or other immunosuppressive agents. However, some patients are refractory to these treatments and others do not tolerate their side effects.

Lymphocyte apoptosis can be induced by several stimuli, such as exposure to ionizing radiation, chemicals or drugs, deprivation of growth factors or stimulation of death receptors, many of whichbelong to the superfamily of receptors of the Tumor Necrosis Factor (TNF). The receptors belonging to this superfamily participate in the control of the immune response by regulating proliferation, differentiation, and survival of immune cells. Among the 26 members belonging to this superfamily, eight contain an intracytoplasmic domain, called Death Domain (DD), which is involved in activation of the caspase signaling pathway leading to cell apoptosis, and work as death receptors. Fas belongs to this group and plays an essential role in the ALPS etiopathogenesis. The interaction of Fas with its ligand, Fas-ligand (Fas-L), induces trimerization of Fas on the cell membrane, which is a critical step for the formation of an intracellular multimolecular complex called “Death Inducing Signaling Complex (DISC)” [22,23], formed by an adapter molecule, FADD (Fas-associating protein with Death Domain or MORT-1), capable of interacting, through its C-terminal portion, with the DD of Fas. Moreover, FADD contains a second interaction domain, called Death Effector Domains (DED) [24], which allows recruitment of procaspase-8, procaspase-10, and cFLIP (FADD-like IL-1β-converting enzyme-inhibitory protein) [8]. Procaspase-8 and -10 are cysteine-protease belonging to the caspase family; they are present as proenzymes in the

cytoplasm and are *self* activated in the context of the DISC through sequential proteolytic cleavage [25].Activated caspase-8 and -10 are then able to activate the executor caspase-3, -7 and -6 triggering cell apoptosis [26,27]. cFLIPis a master anti-apoptotic regulator and resistance factor that suppresses Fas-induced apoptosis by competing with caspase-8 and -10 in DISC formation [28,29].

The ALPS picture is recapitulated in MRL mice homozygous for the *lpr* (lymphoproliferation) or the *gld* (generalized lymphoproliferative disease) characters. Indeed, the molecular defects of the *lpr* and *gld* characters were shown to be a loss of function mutations in the *FAS* and the *FASL* genes respectively [30]. These mice develop a phenotype similar to that shown by ALPS patients, characterized by lymphadenopathy, splenomegaly, autoimmunity with hypergammaglobulinemia, and polyclonal expansion of DN T cells. Moreover, they display production of anti-DNA autoantibodies, glomerulonephritis, arthritis, and vasculitis, which are not typical of ALPS.

The typical *lpr* mutation is a splicing defect and determines reduced expression of Fas in the membrane; a variant mutation, lpr^{cg}, is a point mutation in the DD of Fas which reduces its activity. The *gld* mutation is a point mutation in the C-terminal domain of FasL which reduces its ability to interact with Fas [30]. The genetic background influences the penetrance of these mutations since a severe disease is developed by the MRL mouse strain, while a mild phenotype is developed in the Balb/c background [31,32].

ALPS classification and diagnostic criteria have been recently revised by Oliviera JB et al. (2010) [21].

The diagnostic criteria include two requiredcriteria, two accessory primary criteria, and four accessory secondary criteria (Table 1).

The required criteria are 1) lymphadenopathy and/or splenomegaly, and 2) elevated $TCR\alpha\beta^{+}$-DNT cells in the blood.

The accessory primary criteria are 1) abnormal lymphocyte apoptosis, and2) presence of pathogenic mutations in genes of the FAS pathway.

The secondary accessory criteria are1) presence of elevated blood biomarkers (IL-10, IL-18, VitB12, soluble FasL), 2) characteristic histopathology, 3) combined presenceof autoimmune cytopeniasand polyclonal hypergammaglobulinemia,and 4) family history compatible with ALPS.A definitive ALPS diagnosis is based on presence of bothrequired criteria plusone primary accessory criterion. A probable ALPS diagnosis is based on presence of both required criteria and one secondary accessory criterion (Table 2).

Table 1 Revised diagnostic criteria for ALPS

Required
1. Chronic (> 6 months), nonmalignant, noninfectious lymphadenopathy or splenomegaly or both
2. Elevated $CD3^+TCR\alpha\beta^+CD4^-CD8^-$ DNT cells (≥ 1.5% of total lymphocytes or 2.5% of $CD3^+$ lymphocytes) in the setting of normal or elevated lymphocyte counts
Accessory
Primary
1. Defective lymphocyte apoptosis (in 2 separate assays)
2. Somatic or germline pathogenic mutation in *FAS*, *FASLG*, or *CASP10*
Secondary
1. Elevated plasma sFASL levels (>200 pg/mL) OR elevated plasma interleukin-10 levels (>20 pg/mL) OR elevated serum or plasma vitamin B_{12} levels (> 1500 ng/L) OR elevated plasma interleukin-18 levels > 500 pg/mL
2. Typical immunohistological findings as reviewed by an experienced hematopathologist
3. Autoimmune cytopenias (hemolytic anemia, thrombocytopenia, or neutropenia) AND elevated immunoglobulin G levels (polyclonal hypergammaglobulinemia)
4. Family history of a nonmalignant/noninfectious lymphoproliferation with or without autoimmunity

A definitive diagnosis is based on the presence of both required criteria plus one primary accessory criterion. A probable diagnosis is based on the presence of both required criteria plus one secondary accessory criterion.

Blood.2010 Oct 7;116(14):e35-40. doi: 10.1182/blood-2010-04-280347.

The novel ALPS classification is based on thegenetic defectcausing the disease (Table 2) and includes ALPS-FAS due to mutations of *FAS*, ALPS-FASLG due to mutations of *FASL*, ALPS CASP10 due to mutations of *CASP10*, and ALPS-U due to unknown mutations.

Table 2 Revised classification of ALPS

Previous nomenclature	Revised nomenclature	Gene	Definition
ALPS type 0	ALPS-FAS	*FAS*	Patients fulfill ALPS diagnostic criteria and have germline homozygous mutations in *FAS*.
ALPS type Ia	ALPS-FAS	*FAS*	Patients fulfill ALPS diagnostic criteria and have germline heterozygous mutations in *FAS*.
ALPS type Im	ALPS-sFAS	*FAS*	Patients fulfill ALPS diagnostic criteria and have somatic mutations in *FAS*.
ALPS type Ib	ALPS-FASLG	*FASLG*	Patients fulfill ALPS diagnostic criteria and have germline mutations in FAS ligand.
ALPS type IIa	ALPS-CASP10	*CASP10*	Patients fulfill ALPS diagnostic criteria and have germline mutations in caspase 10.
ALPS type III	ALPS-U	Unknown	Patients meet ALPS diagnostic criteria; however, genetic defect is undetermined (no FAS, FASL, or CASP10 defect).

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Our laboratory, also described patients with lymphadenomegaly/splenomegaly, autoimmune manifestations and defective Fas function, but lacking expansion of DN T cells. This disease has been named Dianzani Autoimmune Lymphoproliferative Disease (DALD) (OMIM reference #605233), and several features indicate that it may have a genetic component in involving the Fas

pathway [33-38]. This disease has been classified as “ALPS-related” disease in the revised ALPS classification.

Apart from the causal mutation, ALPS development and course can be influenced also by other genetic factors influencing the immune response and lymphocyte apoptosis. This may in part explain the great clinical variability of the disease outcome that can be detected not only between patients carrying different mutations, but also between those belonging to the same family and carrying the same mutation. Often the disease is ascribed to heterozygous mutations with incomplete penetrance and generally the parent that carries the mutation is either healthy or displays minor laboratory signs of the disease [8]. Therefore,the disease expression may depend on the co-presence of other genetic or environmental predisposing factors [33].From the point of view of the genetic predisposing factors, our laboratory has previously shown that polymorphic variations of the osteopontin (*OPN*) and perforin (*PRF-1*) genes may be susceptibility factors for ALPS and DALD development and may influence disease expression [34,36]. Osteopontin is a proinflammatory cytokine capable to inhibit lymphocyte apoptosis, whereas perforin is involved in cell-mediated cytotoxicity of NK cells and CTL which are involved not only in the anti-viral immune response but also in the immune response homeostasis.

The research described in this thesis stemmed from these works and analyzed the role played in ALPS and DALD by

1. Variations of the *UNC13D* gene coding for Munc13-4 involved in the perforin function.
2. Variation of the *SAP* gene (SLAM-Associated Protein) involved in NK function.
3. IL-17, a proinflammatory cytokine that may work in tandem with osteopontin.

2.4 Munc13-4

Munc13 proteins are a family of four proteins (Munc13-1, Munc13-2, Munc13-3, Munc13-4) with homology to the protein Unc-13p expressed by Caenorhabditis elegans. The Munc13 proteins generally contain a C1 domain (capable of binding ester phorbol), two C2 domains (capable of binding calcium and phospholipids), and two MHD domains (Munc13-homology-domains) [39]. They regulate, during exocytosis, the formation of a protein complex (trans-SNARE complex) between the membrane of secretory vesicles and the plasma membrane. Munc13-2 is ubiquitously expressed in the body, while Munc13-1 and Munc13-3 are expressed only in the brain. Munc13-1 is expressed by all neurons and plays a role in activation of synaptic exocytosis vesicles; Munc13-3 is expressed in the cerebellum and controls the release of neurotransmitters [40];Munc13-4is involved in degranulation of several immune and non immune cells [39].

UNC13D gene is present on chromosome 17q25, it is long 17 kb (32 exons) and encodes for theMunc13-4 protein consisting of 1088 amino acids. The protein comprises of two MHD and C2 domains but no C1 domain.The MHD domains (F_{557}- I_{677} for MHD1 and E_{788}-K_{894} for MHD2) consist of α-helices with different lengths and amino acid repetitions and are essential for the localization of the protein [39]. The C2 domains (P_{109}-H_{284} for C2A e A_{904}-P_{1047} for C2B) contain five aspartic acid residues forming a site for Ca_{2+} ions that mediate the calcium-dependent interaction with membrane phospholipids. Moreover, the presence of anα-helix in the loop-3 of the C2A domain allows binding with the plasma membrane even in the absence of calcium [39]. Munc13-4 is expressed in cells of the bronchial tissue, spleen, reproductive apparatus and, at a lesser extent, in the cardiac and skeletal muscles, liver, kidney, and brain [39,41].In the immune system, the expression of Munc13-4 is expressed in CTL, NK cells, mast cells, and platelets.In platelets, Munc13-4 is distributed between the cytoplasm and the plasma membrane but is not associated with the dense granules [42]. In mast cells, NK cells and CTL, it is expressed in the granule membrane [39,43] and is involved in the secretion process [44], which consists of several stages: recruitmentof the secretory vesicles toward the immunological synapse and anchorage to the plasma membrane, activation and vesicle fusion, and secretion of the vesicles contents. Rab GTPases are key regulators of the formation, motility, and fusion of the vesicles. Following an activation stimulus (e.g. the binding of the TCR with the MHC plus peptide), lysosomes united to late endosomes lead to the formation of lytic granules that polarize towards the mature immunological synapse [45]. The activation stimulus allows the transition from inactive RabGDP to active RabGTP, promoted by the GEF factor [46,47]. Then, RabGTP interacts with specific effector proteins that allow granule binding to the plasma membrane (anchorage phase). After anchoring,another step (activation) is needed to make the vesicles competent to fuse with the plasma membrane. Munc13-4 is importantin the secretion of the cytotoxic granules in CTL and NK cells butit is not involvedin cytokinesecretion[41]. Moreover, it is involvedin granule secretionin mast cellsandplatelets[39,41].By interacting with Rab27a/GTP, it forms a complex that acts during the stage of vesicle-plasma membrane fusion and is involved in the activation phase [41]. Then, Munc13-4 binds the syntaxin-11/Munc18-2 complex on the plasma membrane and promotes its transition to an active conformation. This change promotes the formation of a structure composed of RabGTP/Slp2_t-SNARE (SNAP23, sintaxin-11/Munc18-2)_v-SNARE (VAMP7) [45]. The complex sintaxin-11/Munc18-2 probably regulates the anchorage of granules and the initial formation of the SNARE complex before the beginning of the activation phase [45]. Following their fusion to the plasma membrane, the granules release their contents in the extracellular microenvironment [41,42,48].

Familial haemophagocytic lymphohistiocytosis (FHL) is a recessive genetic disease due to defective function of perforin-mediated cytotoxicity resulting in ineffective compensatory immune hyperactivation upon viral infection with excessive lymphoproliferation and interferon-γ secretion, causing massive macrophage activation, tissue damage, and fatal outcome. The most frequent genetic causes are mutations of the perforin (FHL2) or Munc13-4 (FHL3) genes, which hit perforin function and its secretion respectively [39]. Patients with FLH3show anormalanchoringof the vesiclesto the plasma membrane, buta defectin the fusionand release of their contents[41].Less frequently FHL can be caused by defective exocytosis of the lytic granules due to mutations of the syntaxin-11 (FLH4) or Munc18-2 (FLH5) genes [40].

2.5 Slam Associating Protein (SAP)

SAP (Slam associating Protein), also called SH2D1A (Src Homology 2 Domain 1A containing protein), is a small intracytoplasmic protein of 15 kDa consisting of a single SH2 domain and a short tail of 28 amino acids at the C-terminus with two phosphorylable tyrosine residues [49]. The *SAP*gene maps on the X chromosome at position Xq25-q26 and comprises four exons and three introns.SAP conforms a family of proteins including also EAT2 (Ewing 's sarcoma-associated trancript-2) and, in rodents, ERT (EAT2-related transducer). Unlike SAP, the other two members are located in tandem on chromosome 1 and probably originated from gene duplication. SAP is expressed on T cells, NK cells, eosinophils, platelets, whereas its expression on B cells is still controversial. SAP is involved in signal transduction by binding to receptors of the SLAM (Signaling Lymphocyte Activation Molecule)family, including six transmembrane receptors belonging to the immunoglobulin superfamily: SLAM (CD150), 2B4 (CD244), Ly -9 (CD229), NK -TB (NTB-A or Ly-108), CD84, and CRACC. With the exception of 2B4, whose ligand is CD48, all the other members of this family of receptors are self-ligands and mediate cell-cell interactions. The expression of these receptors is restricted to the hematopoietic lineage, and differs in the various cell types. In particular, SLAM is present on activated T and B lymphocytes, dendritic cells, monocytes and platelets [50-53]. SLAM is a transmembrane protein of 70 kDa characterized by an extracellular domain composed of two Ig-like motifs that mediate homotypic interactions, a single transmembrane domain, and a long cytoplasmic region characterized by three consensus motifs known as ITSM (immunoreceptor tyrosine-based switch motif-TxYxxV/I) containing phosphorylable tyrosine residues [50,54,55]. In T cells, SLAM triggering by agonist antibodies causes proliferation and production of IFN-γ[56] independently from activation through the TCR. However, SLAM knock-out (KO) mice showed normal IFN-γproduction but reduced secretion of

IL-4 [57].Therefore, SLAM is regarded as a co-stimulatory molecule, whose signal modulates cytokine secretion in T cells [53,55]. SLAM participates in the immunological synapse since it is recruited in proximity to the TCR and is phosphorylated by the tyrosine kinases Fyn and Lck in the ITSM domain (Tyr 281, 307 and 327) upon stimulation of the TCR [54].

SAP and EAT-2 are the main interactors of the SLAM receptor family members; while the binding of EAT-2 to thesereceptors occurs only when the tyrosines are phosphorylated, SAP exhibits the peculiar capacity to associate with SLAM (CD150), but not with other receptors of the family, even in the absence of phosphorylation [54].SAP association with SLAMinvolves the SAP SH2 domain and the ITSM domainsof SLAM; in particular,SAP can bind the motifs containing the phosphotyrosines 307 and 327,whereas the motif containing the tyrosine 281 is bound even in the absence of phosphorylation [54,58]. In addition, SAP can simultaneously bind Fyn, which is required for SLAM activation [59-61]. SAP is able to translocate Fyn in the membrane and quiescent lymphocytes display a stable complex SLAM/SAP/Fyn that,upon stimulation with agonist antibodies, promotesthe phosphorylation of SLAM and the recruitment of other effectormolecules such as inositol phosphatase SHIP-1, Shc adapters, Dok1 Dok2, and guanine exchangers as RasGAP [50,55,62] (Figure 2).

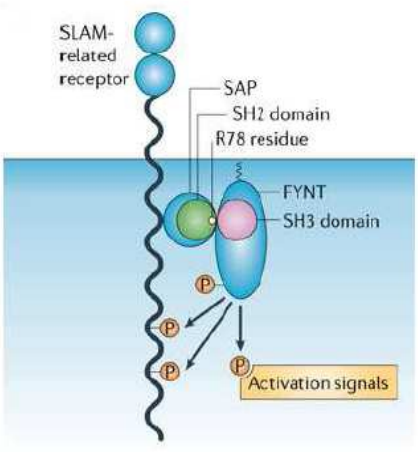

Figure 2. Action mechanism of SAP

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This multiprotein complex promotes the production of cytokines in combination with the signal generated by the TCR. In fact, the signal transduction pathways used by the members of the SLAM family partly overlap with those used by the TCR and reinforce TCR signaling to generate an appropriate immune response[49]. The importance of the signal generated by the SLAM and SAP in the immune response has been underlined by the identification of loss-of-function mutations of SAP in approximately 60% of patients with X-linked lymphoproliferative disease (XLP), a

recessive X-linked genetic disease characterized by an increased and disorganized immune response to Epstein Barr Virus (EBV) infection [63]. The disease is almost asymptomatic in the absence of EBV infection, although in the long term lymphoproliferation, dysgammaglobulinemia and some autoimmune phenomena may nevertheless become evident [64]. These features may be ascribed to defective function of the antiviral cytotoxic response by NK cells and CTL and to altered regulation of the Th response with defective recruitment of Th2 cells in favor of Th1 cells. The defect becomesfully evident following infection of B cells with EBV, resulting in lymphoproliferation and systemic damage, that may be lethal in the pediatric age [50,64,65]. Moreover, Th cells from XLP patients expressed less ICOS and produce a reduced amount of IL-10, which may contribute to the decreased immunoglobulin isotype switchingand the hypogammaglobulinemia detected in these patients [66]. Although mice are not susceptible to EBV infection, SAP KO mice develop functional alterations of the immune system, following viral infection, partly recapitulating XLP features, such as defective plasma cell and memory cell maturation, IgE production, germinal center formation, and excessive, but ineffective, activation of $CD8^+$ lymphocytes [67]. These evidences correlate with altered T cell activation *in vitro*, with deficient secretion of IL-4 and increased production of IFN-γ [68].

SLAM and SAP may also play a role in certain autoimmune diseases since increased expression of SLAM has been detected in T cells from synovial fluid of patients with rheumatoid arthritis, whichmay contribute to increase the production of IL-10, IFN-γ and TNF-αin the autoimmune process [69].Moreover, increased expression of SLAM has been detected in T cells from patients with multiple sclerosis, although the contribution to the development of the disease is still unclear.In MRL*lpr/lpr*mice, loss of expression of SAP attenuatesthe lymphadenopathy and several autoimmune features of the ALPS-like disease displayed by these mice [70].

2.6 Interleukin 17 (IL-17)

Interleukin 17A (IL-17A) is the founding member of a family of proinflammatory cytokines comprising also IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F [71,72].The genes encoding for these cytokine are located in the chromosome 6.IL17A is secreted by several cell types and characterizes the function of Th17 cells, whose differentiation is driven by the expression of the transcription factor ROR-γtinduced upon TCR triggering in the presence of TGF-β, IL- 1β, and IL-6. The expansion of Th17 cells is further supported by IL-23.IL-17A is also secreted by $CD8^+$ T cells, Tγδ cells, NK cells and neutrophils.

IL-17A acts as a potent inflammatory cytokine both *in vitro* and *in vivo* by inducing the expression ofIL-6, IL-1α and TNF-α, several chemokines (suchas KC, MCP-1 and MIP-2) and matrix metalloproteases, which mediatetissue infiltration and tissue destruction [73]. IL-17A is also involved inthe proliferation, maturation and chemiotaxis of neutrophils [74].It activates induction of IL-6, IL-8 and G-CSF in non-immune cells such as fibroblasts and epithelial cells, at least in part through activation of the NF-kB transcription factor [5,21] and the MAPK pathways. In addition,IL-17A costimulates T cells and enhances the maturation of dendriticcells [73]. *In vivo*,mice deficient for the IL-17 receptor (IL-17R) display increased sensitiveness to lungbacterial infections because of reduced recruitment of neutrophils into thelungs [75]. Moreover, overproduction of IL-17A in the lungs leads to increased chemokineexpression and tissue inflammation [76].

A huge bulk of data suggests that Th17 cells are involved in defense against bacterial infection, chronic inflammatory diseases, allergy and autoimmunity.

IL-17A and IL-17F have been implicated in a variety of autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, and psoriasis [73,74,76-86]. They share50% amino acid sequence identity and are secreted as homodimers or IL-17A/F heterodimers [76,77,87-91]. The crystal structure of IL-17F shows that it forms a disulfide-linked dimer that contains a cysteine knot motif similar to that reported for members of the nerve growth factor and the TGF superfamilies [92-94]. Given the high degree of amino acid homology between IL-17A and IL-17F and the conservation of the four cysteines that form the knot, it is likely that IL-17A and IL-17F adopt a similar structure[95-97].

The members of the IL-17 cytokine family interact with a family of IL-17 receptors (IL-17R), comprising IL-17RA, RB, RC, RD, and RE. Despite considerable sequence divergence, several genes encoding for these receptors are clustered in the human chromosome 3 (for IL-17RB, IL-17RC, IL-17RD and IL-17RE), or the mouse chromosomes 6 (IL-17RA, IL-17RC and IL-17RE) and 14 (IL-17RB and IL-17RD). These receptors can interact with each other to form multimeric complexes binding different cytokines of the IL-17 family and can be expressed by several cell types, such as epithelial cells, fibroblasts, neutrophils, T and B lymphocytes (Table 3). Similarly to other cytokine receptor complexes, such as the TNF and the Toll-like receptors, these complexes are pre-assembled in the plasma membrane, which allows a rapid and specific response upon binding to their ligand cytokine.

IL-17RA binds IL-17A with high affinity and IL-17F with low affinity, which suggests that both IL-17A and IL-17F utilize IL-17RA as a part of their receptor complex; this complex undergoes a conformational change upon binding of the IL-17A or IL-17F homodimers or the IL-17AF heterodimer. These dimers bind, with similar affinities, also IL-17RC, that seems to work as a co-

receptor with IL-17RA for IL-17A and IL-17F signaling. IL-17RA can interact also with IL-17RB to bind to IL-25 involved in allergic disease and defense against helminthic parasites, but IL-17RB can bind also IL-17B and the IL-17E/IL-25 heterodimer. IL-17RD too can interact with IL-17RA, although the biological significance of this association remains unclear, whereas IL-17RE is known to bind IL-17C (Table 3) [98].

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	Other common names	Receptor(s)	Main functions	Expression
IL-17	IL-17A, CTLA-8	IL-17RA IL-17RC	Autoimmune pathology, Neutrophil recruitment, immunity to extracellular pathogens	Th17, CD8 cells, γδ-TCR+ T cells, NK, NKT, LTi
IL-17B		IL-17RB	Pro-inflammatory activities?	GI tract, pancreas, neurons
IL-17C		IL-17RE	Pro-inflammatory activities?	Prostate, fetal kidney
IL-17D		?	Pro-inflammatory activities?	Muscle, brain heart lung, pancreas, adpose tissue
IL-17E	IL-25	IL-17RB IL-17RA	Induce Th2, suppress Th17	Intraepithelial lymphocytes, lung epithelial cells, alveolar macrophages, eosinophils, basophils, NKT cells, Th2 cells, mast cells, GI tract, uterus
IL-17F		IL-17RA IL-17RC	Neutrophil recruitment, immunity to extracellular pathogens	Th17, CD8 cells, γδ-TCR+ T cells, NK, NKT, LTi
IL-17A/F		IL-17RA IL-17RC	Autoimmune pathology (presumed), Neutrophil recruitment, immunity to extracellular pathogens	Th17, CD8 cells, γδ-TCR+ T cells, NK, NKT, LTi
vIL-17	ORF13	IL-17RA IL-17RC?	unknown	*Herpesvirus saimiri*

Table 3. Extended IL-17/IL-17R family, expression and known functions

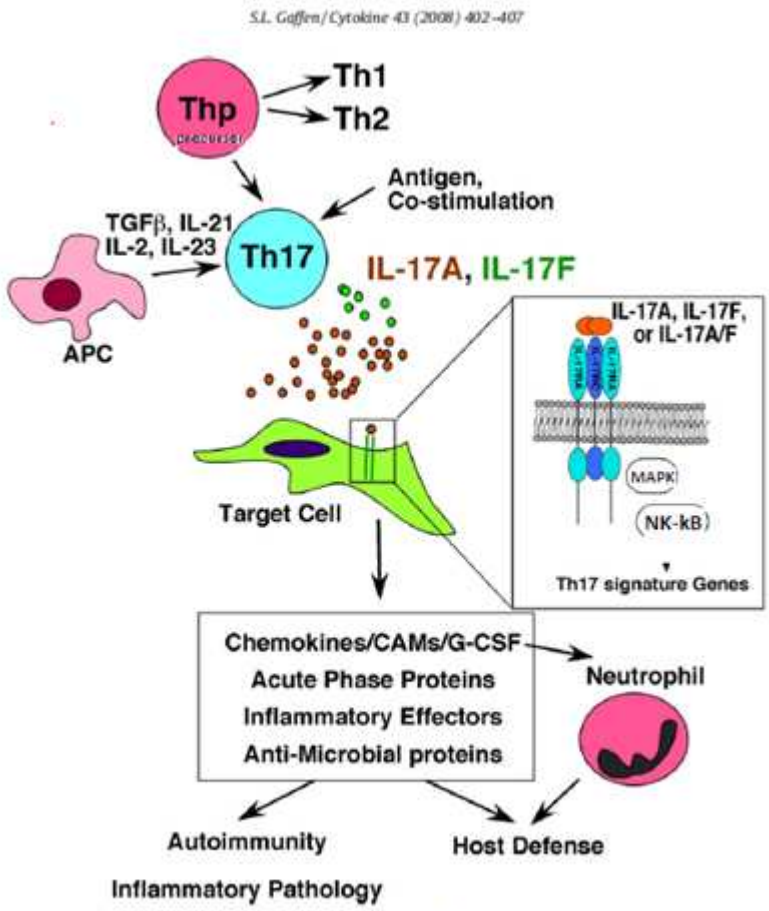

Figure 3. Th cell differentiation and IL-17 functions

Several studies in the late 1990s implicated IL-17 in the pathogenesis of autoimmunity. Elevated IL-17 levels were found in rheumatoid arthritis, systemic lupus erytemathosus, psoriasis, inflammatory bowel disease and vasculitis. In particular, patients with systemic lupus erythematosus display high serum levels of IL-17A and IL-23 and increased numbers of IL-17-producing T cells in the peripheral blood, and these high levels correlate with disease activity. Moreover, some SLE patients show expansion of DN T cells, a hallmark of ALPS, secreting high levels of IL-17A. Moreover, high levels of IL-17A are produced by DN T cells that infiltrate the nephritic kidneys in MRL*lpr/lpr* mice.

Rodent models of rheumatoid arthritis, such as collagen-induced arthritis, indicated that IL-17 might play a key role in the disease pathogenesis. Conversely, antibodies to IL-17 dramatically reduced inflammation and bone erosion in CIA [84,99] and IL-17A KO mice are resistant to collagen-induced arthritis[85]. Treatments with anti-IL-17A antibodies were found to be protective also in a mouse model of Crohn's disease and inExperimental Autoimmune Encephalomyelitis, a mouse model of MS.

3. ARTICLE 1:

Variations of the UNC13D gene in patients with autoimmune lymphoproliferative syndrome

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PLOS ONE

Variations of the *UNC13D* Gene in Patients with Autoimmune Lymphoproliferative Syndrome

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Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is caused by genetic defects decreasing Fas function and is characterized by lymphadenopathy/splenomegaly and expansion of CD4/CD8 double-negative T cells. This latter expansion is absent in the ALPS variant named Dianzani Autoimmune/lymphoproliferative Disease (DALD). In addition to the causative mutations, the genetic background influences ALPS and DALD development. We previously suggested a disease-modifying role for the perforin gene involved in familial hemophagocytic lymphohistiocytosis (FHL). The *UNC13D* gene codes for Munc13-4, which is involved in perforin secretion and FHL development, and thus, another candidate for a disease-modifying role in ALPS and DALD. In this work, we sequenced *UNC13D* in 21 ALPS and 20 DALD patients and compared these results with sequences obtained from 61 healthy subjects and 38 multiple sclerosis (MS) patients. We detected four rare missense variations in three heterozygous ALPS patients carrying p.Cys112Ser, p.Val781Ile, and a haplotype comprising both p.Ile848Leu and p.Ala995Pro. Transfection of the mutant cDNAs into HMC-1 cells showed that they decreased granule exocytosis, compared to the wild-type construct. An additional rare missense variation, p.Pro271Ser, was detected in a healthy subject, but this variation did not decrease Munc13-4 function. These data suggest that rare loss-of-function variations of *UND13D* are risk factors for ALPS development.

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Introduction

The lytic granules of cytotoxic T cells (CTL) and natural killer (NK) cells contain perforin and granzymes, which are released on the target cell surface and induce its death[1]. . The exocytosis mechanism of the lytic granules is not fully understood, but it involves machinery composed of several proteins including Munc13-4, Munc18-2, and syntaxin11 [2]. Deficiencies of perforin function are responsible for familial hemophagocytic lymphohistiocytosis (FHL), an autosomal recessive disease characterized by bouts of prolonged fever, hepatosplenomegaly, and cytopenia due to defective function of CTL and NK cells. FHL has been ascribed to defective clearance of virus-infected cells leading to cytokine and effector cell overproduction with massive tissue damage [3]. Approximately 40% of FHL cases (FHL2) are due to mutations of the perforin gene (*PRF1*), with another 40% (FHL3) due to mutations of the Munc13-4 gene (*UNC13D*). Moreover, a small number of patients with FHL have been found to harbor mutations of *STX11*, encoding Syntaxin-11 (FHL4), or *STXBP2*, encoding Munc18-2 (FHL5) [4].

Autoimmune lymphoproliferative syndrome (ALPS) is another genetic lymphoproliferative disease and is characterized by lymphadenomegaly and/or splenomegaly, due to polyclonal accumulation of lymphocytes, and peripheral expansion of CD4/CD8 double-negative (DN) T cells [5–10]. In addition, patients often display autoimmune manifestations that predominantly involve blood cells and are predisposed to lymphomas in adulthood [11]. ALPS is due to defective function of the Fas/Apo-1 (CD95) death receptor, inducing apoptosis of the Fas-expressing cell upon binding with Fas ligand (FasL) [12,13]. Activated lymphocytes express Fas and the Fas/FasL interaction is involved in shutting off immune responses, lymphocyte lifespan regulation, and maintenance of peripheral tolerance [14,15]. Moreover, the Fas pathway is an additional weapon, reinforcing the perforin system in the cytotoxicity mediated by CTL and NK cells because these cells express FasL and induce apoptosis of target cells expressing Fas. In most patients (ALPS-FAS), ALPS is due to mutations of the Fas gene (*FAS*), but a small number of patients (ALPS-FASL and ALPS-CASP10) carry mutations in the genes encoding FasL (*FASL*) or caspase-10 (*CASP10*), a downstream effector in the Fas/FasL pathway. As a substantial proportion of ALPS patients (ALPS-U) lack mutations in *FAS*, *FASL*, and *CASP10*; it seems likely that mutations in unknown genes encoding other downstream components of the Fas cell death pathway may give rise to the additional ALPS cases [9,10]. We have also described an incomplete form of ALPS showing defective Fas function, autoimmunity, and lymphoproliferation, but lacking the expansion of DN T cells. This variant form has been named Dianzani Autoimmune Lymphoproliferative Disease (DALD) by Victor McKusick (OMIM # 605233) [10,16–18]. Patients with DALD did not display mutations in *FAS*, *FASL*, or *CASP10*, but most of the parents displayed a defect in the Fas pathway. These data suggest that mutations in genes encoding downstream effectors of the Fas pathway may also give rise to DALD.

In addition to the Fas defect, the clinical presentation of ALPS also appears to be influenced by modifier genes. In mice, a disease displaying features of ALPS has been reported for MRL *lpr/lpr* and *gld/gld* mice, carrying mutations of *FAS* and *FASL*, respectively. Disease presentation in these mice is dramatically affected by strain background, with strains other than MRL showing much milder phenotypes when homozygous for either *lpr* or *gld* mutations [12,19]. Similar background effects likely explain the incomplete penetrance of ALPS mutations in humans [20]. Most ALPS patients are heterozygous for the *FAS* mutation, but parents carrying the same mutation are generally healthy. The same observation is true in DALD, where parents typically display defective Fas function, but are otherwise healthy [17,18]. This observation indicates that mutations in genes of the Fas pathway may be necessary but not sufficient for ALPS development and variations in one or more additional genes may influence disease presentation [9].

In previous works, we correlated certain variants of the perforin gene (*PRF1*) with ALPS/DALD development and suggested that mild heterozygous variations of *PRF1* incapable of inducing FHL may act as susceptibility genes for ALPS/DALD development in subjects displaying defective Fas function [21,22]. The aim of this work was to extend this observation to the *UNC13D* gene, looking for variations in ALPS and DALD patients and assessing its potential role as a disease-modifier gene. We found that loss-of-function variations of *UNC13D* are relatively frequent in patients with ALPS, suggesting that it may influence the presentation of this lymphoproliferative disorder.

Materials and Methods

Patients

We analyzed 41 unrelated Italian patients, 21 with ALPS and 20 with DALD. All patients were diagnosed at the Pediatric Department of the University of Turin using criteria established at the 2009 ALPS NIH International Workshop [10]. *FAS* (NCBI ID: 355) and *CASP10* (ID: 843) were sequenced in all patients as reported previously [16,17]. Among the ALPS patients, 7 carried heterozygous mutations of *FAS* (ALPS-FAS), 14 did not carry any known mutation (ALPS-U). None of the patients fulfilled the diagnostic criteria for FHL. A total of 61 healthy individuals were used as controls for *UND13D* sequencing, and a second cohort of 100 healthy controls were used to genotype the rare variations. Moreover, *UNC13D* was sequenced in 38 patients with Multiple Sclerosis (MS) from the MS Center of the "Amedeo Avogadro" University of Eastern Piedmont (Novara). The study was planned according to the guidelines of the local ethical committee, Azienda Ospedaliera della Carità, of Novara that approved the study (Protocol 106/CE). For the patients followed at Paediatric Department of the University of Torino, a written informed consents was signed by the patients, or by the parents if they were minors.

Fas function assay

Fas-induced cell death was evaluated on T cells obtained by activating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (Sigma, St Louis, MO, Canada) at days 0 (1 µg/mL) and 12 (0.1 µg/mL) and cultured in RPMI 1640 plus 10% fetal calf serum and recombinant IL-2 (rIL-2, 2 U/mL) (Sigma). Fas function was assessed 6 days after the second stimulation (day 21). Cells were incubated with control medium or anti-Fas monoclonal antibody (mAb) (CH 11, 1 µg/mL) (Millipore, Billerica, MA) in the presence of rIL-2 (1 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting live cells by the trypan blue exclusion test. Assays were performed in duplicate. Cells from 2 healthy donors were included in each experiment as positive controls. The results were expressed as percent specific cell-survival, calculated as follows: (total live cell count in the assay well/total live-cell count in the control well) X100%. Fas function was defined as defective when cell survival was less than 82% (the 95th percentile of data obtained from 200 healthy controls) [17,18].

UNC13D sequencing

Genomic DNA was isolated from peripheral blood samples using a BioRobot® EZ1 Workstation (Qiagen, Jesi, Italy).

Exons and intron-exon boundaries of *UNC13D* (ID. 201294), were amplified and directly sequenced in both directions with the BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Primers are available upon request. Sequences were analyzed and compared with the reported gene structure. The missense variations identified in patients were also assessed in parents.

Allele expression was evaluated in total RNA extracted from PBMC of heterozygous donors. RNA was reverse transcribed into cDNA with the ThermoScript™ RT PCR System (Invitrogen, Burlington, ON, Canada) and the exons containing the *UNC13D* variations were amplified. PCR products were subcloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA) and transformed into TOP10 *E. coli* competent cells (Invitrogen). In each selected patient, we screened 30 independent colonies; plasmid DNA was extracted with a QIAPrep Spin miniprep Kit (QIAGEN GmbH, Hilden, Germany) and sequenced.

Functional analysis of the variations

Munc13-4 cDNA (ImaGenes, BioDiscovery, Inc. Suite CA, USA) was subcloned into the pcDNA 3.1 expression vector (Invitrogen), and the Sv5 tag was added at the 5' end by PCR. The mutants were created in the Munc13-4 wild-type construct by PCR and then transfected into the HMC-1 human mast cell line, originally established from the peripheral blood of a patient with mast cell leukemia [23] and kindly provided by C. Dianzani. Cells were transfected by Amaxa Cell Line Nucleofactor Kit V (Lonza, Basel, Switzerland), according to the manufacturer's instructions. Briefly, 4 µg of each construct were co-transfected with 1 µg of the pEGFP vector (Invitrogen). Transfection efficiency was analyzed by cytofluorimetric evaluation, and determined by calculating the % of GFP expressing cells. To investigate Munc13-4 expression levels, cells were lysed and proteins resolved by gel electrophoresis were analyzed using mAb to Sv5 and actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Immunoreactive proteins were visualized with HRP-conjugated goat anti-mouse IgG (Sigma).

To investigate primary granule exocytosis, transfected HMC-1 cells (1x10^6/ml) were incubated in Tyrod Buffer (Hepes 10 mM pH−7.4, NaCl 173 mM, KCl 2.9 mM, $NaHCO_3$ 12 mM with 1.6 mM $CaCl_2$ and 5 mM Glucose) and stimulated with 10^{-6} M formyl-methionyl-leucyl-phenylalanine peptide (fMLP, Sigma) at 37°C for 10 min. Exocytosis was then assessed on the GFP-expressing cells by cytofluorimetric analysis of CD63 expression, using the mean fluorescence intensity ratio between stimulated and unstimulated cells set at 100% [21].

The functional effects of the *FAS* mutations were assessed by transfecting the mutated cDNA, subcloned into pcDNA3.1 (Invitrogen) in 293T cells. Wild-type cDNA of *FAS* bearing the FLAG tag at the 5'-end was a kind gift of Giovina Ruberti (National Research Council, CNR, Rome). The p.Gln273His and p.Glu261Lys mutants were created in the *FAS* construct by PCR. Cells were transfected and lysed as for Munc13-4 and immunoblotted with anti-FLAG mAb (Sigma).

Analysis of caspase-8 activity

Caspase-8 activity was evaluated, as previously reported [25], in 100 µg of cell lysates obtained from Fas-transfected 293T cells (5 x 10^6), 24 hours after transfection, using a fluorimetric assay according to the manufacturer's instructions (MBL, Watertown, MA). The results were expressed as relative caspase-8 activity (in %), which was calculated as (activity of Fas transfected cells/activity of mock transfected cells) X 100%.

Functional analyses of patient NK cells

PBMC were cultured overnight at 37°C in 5% CO_2 in media with or without rIL-2 (600 UI/ml) (Proleukin, Chiron Corp., Emeryville, USA) to test degranulation of resting and activated NK cells, respectively. PBMC derived from patients' relatives and/or unrelated healthy donors were tested in parallel. Surface expression of CD107a was assessed on CD3$^-$CD56$^+$ cells upon co-incubation of PBMC with K562 cells in the presence of Phycoerythrin-conjugated anti-CD107a mAb for 2 hours at 37°C, as previously described [26,27]. Thereafter, cells were stained with APC conjugated anti CD56 and PerCP conjugated anti-CD3 mAb, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, CA, USA). The results were considered by assessing the change in % CD107a (i.e., % CD107a$^+$ cells in stimulated samples – % CD107a$^+$ cells in unstimulated samples). All reagents were from BD Biosciences. NK cells were also purified using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada), following manufacturer's instructions, and cultured in appropriate conditions to obtain high numbers of polyclonal activated NK cells [26]. To analyze the cytolytic activity in 4 hour ^{51}Cr-release assays, PBMC were tested against K562 cells, while activated NK cells were tested against the HLA-class I-negative B-EBV cell line 721.221, as previously described; lytic units were calculated at 30% lysis [26,27].

Statistical analysis

Statistical analysis was performed using the ANOVA followed by Dunnett's multiple comparison test; *p<0.05, **p<0.01. The results are shown as the mean and standard error (SE). Genotype distributions were analyzed with the Fisher's exact test. All P-values are 2-tailed, and the significance cut off was p<0.05.

Results

Genetic analyses

The coding sequences (exons and intron boundaries) of *UNC13D* were sequenced in 21 patients with ALPS (ALPS-FAS: N=7; ALPS-U:N = 14) and 20 with DALD. We identified 6 heterozygous missense variations in *UNC13D* in 8 patients (2 ALPS-FAS, 3 ALPS-U, 3 DALD). The variations and their inheritance are described in Table 1 and Figure 1.

Two variations had been previously described in patients with FHL; two patients carried p.Ala59Thr (c.175G>A; rs9904366) and three p.Arg928Cys (c.2782C>T; rs35037984). Four other variations were identified, i.e., p.Cys112Ser (c.335G>C, rs141540493), p.Val781Ile (c.2342G>A,

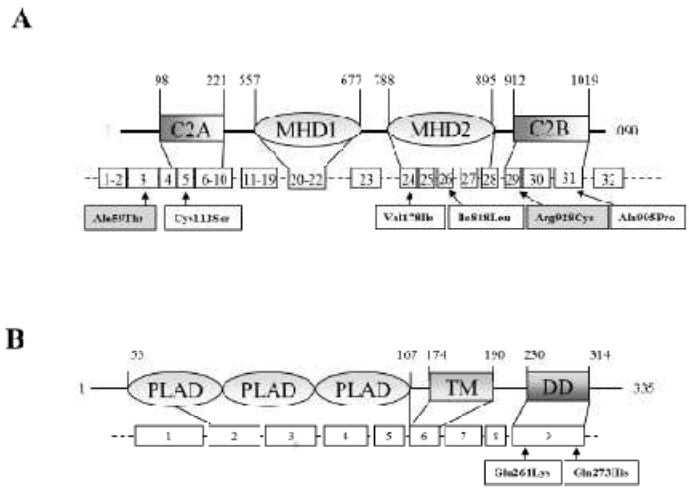

Figure 1. *UNC13D* and *FAS* variations carried by ALPS/DALD patients. Graphical representation (not in scale) of the Munc13-4 [A] and Fas [B] proteins (upper schemes: numbers indicate the amino acid positions) and genes (lower scheme: boxes represent the exons, arrows indicate the mutations). C2: C2 domain, MHD: Munc13-homology domain, PLAD: preligand assembly domain; TM: transmembrane domain; DD: death domain.

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Table 1. Gene variations detected in patients with ALPS or DALD.

Patients (gender)	Diagnosis		Fas function*		*FAS*		*UNC13D*	
	Pt	Pt	F	M	Variation	Inh†	Variation	Inh†
Pt.1 (female)	ALPS-FAS	D	D	N	p.Gln273His	F	p.Arg928Cys	M
					(c.819G>C)		(c.2782C>T)	or F‡
Pt.2 (male)	ALPS-FAS	D	ND	ND	p.Glu261Lys	F	p.Cys112Ser	M
					(c.755G>A)		(c.335C>G)	
Pt.3 (female)	ALPS-U	D	D	D			p.Ala59Thr	M
							(c.175G>A)	
Pt.4 (male)	ALPS-U	D	D	D			p.Ile848Leu	M
							(c.2542A>C)	
							p.Ala995Pro	M
							(c.2983G>C)	
Pt.5 (female)	ALPS-U	D	ND	ND			p.Val781Ile	ND
							(c.2342G>A)	
Pt.6 (male)	DALD	D	D	D			p.Ala59Thr	F
							(c.175G>A)	
Pt.7 (female)	DALD	D	D	D			p.Arg928Cys	M
							(c.2782C>T)	
Pt.8 (male)	DALD	D	D	ND			p.Arg928Cys	ND
							(c.2782C>T)	

* D = defective, N = normal. Pt = patient, F = father, M = mother.

‡ Inheritance, F = father, M = mother, ND = not determined, no parent displayed ALPS, DALD, XLP, or FHL. Pt.1's mother had rheumatoid arthritis.

† both parents carried the variation.

Table 2. Missense variations detected in 21 ALPS, 20 DALD, 38 MS patients, and 61 healthy controls.

	Functional effect‡	ALPS (N=42)*	DALD (N=40)*	Controls (N=122)*	MS (N=76)*
Frequent variations†					
Arg928Cys	Not performed	1	2	8	2
Ala59Thr	Not performed	1	1	5	3
Total alleles with frequent variations		2	3	13	5
Private variations†					
Ile848Leu‡	Loss-of-function‡	1	0	0	0
Ala995Pro‡	Loss-of-function‡	1	0	0	0
Cys112Ser	Loss-of-function	1	0	0	0
Val781Ile	Loss-of-function	1	0	0	0
Pro271Ser	Normal Function	0	0	1	0
Total alleles with loss of function		3	0	0	0
p-0.03§					

* allele numbers

† amino acid substitution

‡ carried in the same allele

§ P value vs Controls (Fisher exact test)

rs149871493), p.Ile848Leu (c.2542A>C; rs144963313), and p.Ala995Pro (c.2983G>C; rs138760432). These variations have been recently described in the dbSNP database as rare variants with an allele frequency of <0.01%, and each variant was found in a single patient. Pt. 4 carried two variations, p.Ile848Leu and p.Ala995Pro, inherited from the same parent.

To assess the variation frequency in the general populations and in subjects with a different autoimmune disease, we sequenced *UNC13D* in 61 healthy controls and 38 patients with MS. The results showed that p.Ala59Thr was found in 5 healthy controls and 3 MS patients, and p.Arg928Cys in 8 healthy controls and 2 MS patients. Moreover, one healthy control carried the novel variation p.Pro271Ser (c.811C>T), absent in the other groups (Table 2). Because p.Ala59Thr and p.Arg928Cys were detected in all patients and control groups with similar allelic frequencies, they were not further considered. Because p.Cys112Ser, p.Val781Ile, p.Ile848Leu, and p.Ala995Pro were detected in the ALPS group alone, we further assessed their frequency in the Italian population by genotyping them in 100 additional healthy controls. None of these variations were identified in the healthy controls, indicating that their allele frequency is relatively low.

Of the five patients whose inheritance pattern of *UNC13D* variations could be determined, four (80%) were maternal and one (20%) was paternal (Table 1). To evaluate whether this apparent bias was due to genetic imprinting favoring expression of the maternal allele, we performed RT-PCR on mRNA derived from Pt.2, Pt.7, and two other patients who were heterozygous for the common synonymous polymorphism c.3198A>G (p.Gln1066Gln). Complementary DNA were then cloned, and 30 independent clones were sequenced for each patient. The results showed that both alleles were expressed at approximately the same levels in each subject, which did not support maternal genetic imprinting in these patients (data not shown).

Functional analyses

Fas-induced cell death assessed in T cells from the ALPS and DALD patients carrying the *UNC13D* variations is shown in Figure 2A. All patients displayed defective Fas function except for Pt.1, whose Fas function was considered as borderline with regard to statistical significance.

The *FAS* mutations present in Pt.1 and Pt.2 were p.Gln273His and p.Glu261Lys missense mutations, respectively. To assess their effect on Fas function, the wild-type (Fas^{WT}) and mutated ($Fas^{Gln273His}$, $Fas^{Glu261Lys}$) forms of Fas cDNA were transfected into 293T cells. Twenty four hours after transfection, 293T cells were harvested and caspase-8 activity was assessed in cell lysates. The results showed that caspase-8 activation was significantly lower in the cells transfected with $Fas^{Gln273His}$ and $Fas^{Glu261Lys}$ than in those transfected with Fas^{WT} (Figure 2B).

The effects of the *UNC13D* missense variations on Munc13-4 protein expression and NK function were evaluated in the PBMC of all patients. NK function was evaluated by assessing the cytotoxic activity of resting NK cells against K562 cells and that of activated NK cells against the HLA-class I-negative B-EBV cell line 721.221. Resting and activated NK cells were also tested for granule exocytosis, the most appropriate assay to detect Munc13-4 defects [28]. The results showed that values were in the normal range for all patients (data not shown), which was consistent with previous data on donors carrying heterozygous mutations of *UNC13D*.

To further assess the functional effect of the *UNC13D* variations detected in one subject only, they were inserted into a cDNA encoding *UNC13D* fused to the SV5 epitope tag. The $Munc13\text{-}4^{wt}$, $Munc13\text{-}4^{Cys112Ser}$, $Munc13\text{-}4^{Val781Ile}$, $Munc13\text{-}4^{Ile848Leu}$, $Munc13\text{-}4^{Ala995Pro}$, $Munc13\text{-}4^{Ile848Leu/Ala995Pro}$, and $Munc13\text{-}4^{Pro271Ser}$ constructs were transfected into the HMC-1 mastocytoma cell line. Western blot analysis showed that all constructs were expressed at similar levels indicating that the polymorphisms did not have a substantial effect on Munc13-4 expression (data not shown). To assess the effect of these variations on Munc13-4 function, we evaluated the capacity of fMLP to induce secretory granule fusion with the plasma membrane in HMC-1 cells. Fusion was monitored by an increase in CD63 expression on the cell surface. Figure 3 shows that fMLP increased surface expression of CD63 by similar amounts in the cells transfected with $Munc13\text{-}4^{wt}$ or $Munc13\text{-}4^{Pro271Ser}$ constructs. In contrast, fMLP increased CD63 expression to a significantly lower extent in cells that have been transfected with the $Munc13\text{-}4^{Cys112Ser}$, $Munc13\text{-}4^{Val781Ile}$, $Munc13\text{-}4^{Ile848Leu}$, $Munc13\text{-}4^{Ala995Pro}$, and $Munc13\text{-}4^{Ile848Leu/Ala995Pro}$ constructs (*$p<0.05$).

Discussion

Munc13-4 is a member of the Munc13-like family of proteins. It is highly expressed in CTL, NK cells, and mast cells and it is involved in granule exocytosis. Once granules are tethered to the plasma membrane, a priming step is required to enable

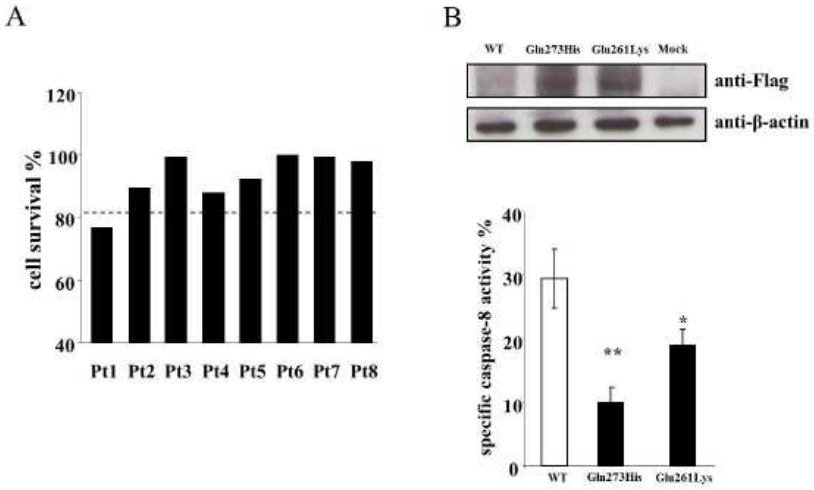

Figure 2. Defective Fas function in the ALPS and DALD patients carrying the UNC13D variations. [A] Fas-induced cell death in T cells from the ALPS and DALD patients carrying the *UNC13D* variations. Activated T cells were treated with anti-Fas mAb and survival was assessed after 18 hours. The results are expressed as specific cell survival %. The dotted line indicates the upper limit of the normal range calculated as the 95th percentile of data obtained from 200 healthy controls; two or more were run in each experiment as positive controls; each patient was evaluated at least twice with the same result. [B] Fas expression and caspase-8 activity in lysates of 293T cells transfected with the wild-type (WT) or mutated form of *FAS* (Pt.1: p.Gln273His, Pt.2: p.Glu261Lys); cells were lysed 24 hours after transfection. *Upper panels*: Western blot analysis of the transfected Fas performed using anti-FLAG and anti-β-actin antibodies. *Lower panels*: fluorimetric enzyme assay for caspase-8 activity. Data are relative to those displayed by mock-transfected cells and are expressed as the mean and SE of the results from 4 experiments performed in duplicate. *p<0.05; **p<0.01 vs. Fas^{wt} transfected cells.

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fusion of the granule membrane with the plasma membrane. In this priming step, granules interact with a docking complex composed of Munc18-2 and Syntaxin-11. Thus, Munc13-4 triggers the switch of syntaxin-11 from a closed to an open conformation enabling fusion [29].

The present study detected six missense variations of *UNC13D* in ALPS-FAS (2/7, 29%), ALPS-U (3/14, 21%), and DALD (3/20, 15%) patients. Among them, two (p.Ala59Thr, p.Arg928Cys) had been previously reported in FHL3, whereas the other four (p.Cys112Ser, p.Val781Ile, p.Ile848Leu, p.Ala995Pro) were reported in the dbSNP database as rare variations with unknown functional and pathological significance. Moreover, both Ile848Leu and Ala995Pro have been described in *cis* in one patient with systemic Juvenile Idiopathic Arthritis (SJIA) and patients with FHL [30].

Only p.Ala59Thr and p.Arg928Cys were found in more than one patient, with the former carried by an ALPS-U and a DALD patient, and the latter by an ALPS-FAS and two DALD patients. These p.Ala59Thr and p.Arg928Cys variations were detected in several healthy controls and MS patients with similar allelic frequencies (p.Ala59Thr: ALPS 2.4%, DALD 2.5%, healthy controls 4.1%, MS 4%; p.Arg928Cys: ALPS 2.4%, DALD 5%, healthy controls 6.5%, MS 2.6%). These data argue against substantial role for these variations in ALPS and DALD. The p.Ala59Thr variation had been previously reported in two families with FHL, but always in *cis* with a pathogenic mutation, making it difficult to assess its contribution to pathogenesis [31]. The p.Arg928Cys variation had been previously reported in FHL patients and a recent genotype-phenotype study detected it in 8 patients carrying biallelic *UNC13D* mutations from 7 unrelated families [27]; yet, some of these FHL3 patients had a third missense mutation too. However, it could be a mild variant whose effect could not be detected in the small groups of subjects used in our study.

The other four variations were carried by three ALPS patients and were absent in DALD and MS patients, and in the healthy controls. The p.Cys112Ser variation was detected in an ALPS-FAS patient who also carried a *FAS* mutation (p.Gln261Lys); the *FAS* and the *UNC13D* mutations were inherited from the father and the mother, respectively. The p.Ile848Leu and p.Ala995Pro variations were carried by an ALPS-U patient and were in *cis*, as previously reported in SIJA and FHL [30], because both of them were inherited from the mother. The p.Val781Ile variation was detected in an ALPS-U patient. These variations were located within key functional domains of Munc13-4, characterized by two C2 domains (C2A, C2B) separated by long sequences containing two Munc13-homology domains (MHD) [29,32–34]. C2 domains bind calcium ions and are involved in targeting proteins to cell membranes; MHD domains are essential for the cellular localization of Munc13-4. The p.Cys112Ser variation was located in the C2A domain, p.Val781Ile and p.Ile848Leu in the

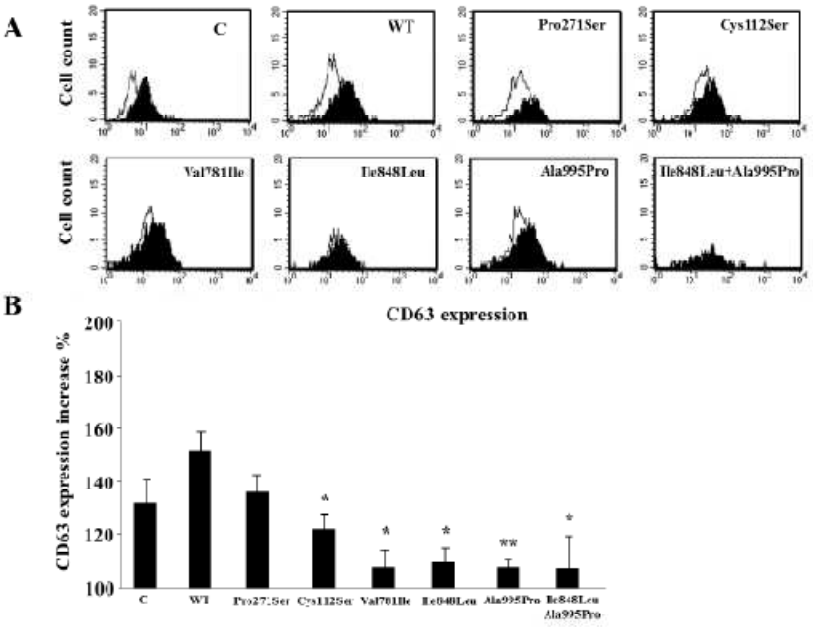

Figure 3. Functional effect of the "private" missense variations of *UNC13D*. HMC-1 cells were transiently transfected with wild-type (WT) *UNC13D* and mutated forms carrying the (p.Cys112Ser, p.Val781Ile, p.Ile848Leu, p.Ala995Pro, p. Ile848Leu/ p.Ala995Pro, and p.Pro271Ser) variations (C = untransfected cells). Twenty-four hours after transfection, cells were tested (or not) for 10 min with fMLP, and expression of CD63 was evaluated by flow cytometry. [A] Cytofluorimetric histograms of CD63 expression in fMLP-stimulated (black) and unstimulated (white) cells transfected with each construct; one experimental representative of six is shown. [B] Mean and SE of the fMLP-induced expression of CD63 from six experiments; results are relative to the CD63 expression displayed by unstimulated cells (set at 100%) in each experiment; the asterisk marks the statistically significant difference versus cells transfected with the WT form; *p<0.05; **p<0.01 vs MUNC^{wt} transfected cells.

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MHD2 domain, and p.Ala995Pro in the C2B domain. The effect of these variations was assessed upon transfection in the HMC-1 mast cell line, commonly used to study granule exocytosis, which showed that all of them significantly decreased Munc13-4 function as detected by decreased fMLP induced granule exocytosis. By contrast, one further variation (p.Pro271Ser) detected in a healthy control was located in the C2A domain but functional analysis showed that it did not significantly decrease Munc13-4 function.

The loss of function effects detected in the transfected cells are in contrast with the normal NK activity detected in the PBMC of the patients carrying the p.Cys112Ser, p.Val781Ile, p.Ile848Leu, and p.Ala995Pro variation. This discrepancy may be ascribed to a difference in sensitivity of the two types of assays. The NK function assays can, in fact, detect severe defects displayed by subjects carrying biallelic loss of function mutations of *PRF1* or *UNC13D*, but not the mild defect displayed by their healthy parents carrying monoallelic mutations. By contrast, mild defects could be detected by our assay in HMC-1 cells in which transfection forces expression of high levels of the Munc13-4 variants.

These data showed that rare loss of function variations of *UNC13D* are observed in ALPS patients with a higher frequency (7%) than in the healthy control (0%), DALD (0%), and MS (0%) groups. Thus, these variations may have an impact in the development of ALPS. Support for this hypothesis comes from a patient who carried loss-of-function mutations in *FAS*, *UNC13D*, and *XIAP*. He was not included in this study because the genetic and clinical complexity of his picture fulfilled the diagnostic criteria of ALPS but also shared features of FHL and X-linked lymphoproliferative disease (Boggio E. et al, submitted).

Defective functions of Fas and Munc13-4 might cooperate in disrupting the ability of the immune system to shut off and interfere with the anti-viral response. These processes involve both Fas and NK/NKT cell function whose cytotoxicity is crucial for the clearance of virus-infected cells and the fratricide of activated immune cells [35]. Persistence of viral infection and an inability to switch off the immune response may contribute to the lymphocyte accumulation and the autoimmune reactions displayed by ALPS patients.

These data suggest that the *UNC13D* variations may be considered part of an oligogenic background, predisposing individuals to ALPS development. This may involve genes encoding for perforin (*PRF1*), osteopontin (*OPN*), and Signaling Lymphocyte Activation Molecule-Associated Protein

(*SH2D1A*), whose variations have been suggested to be risk factors for ALPS or DALD development. From this perspective, patients 1, 3, 4, 5, 6, 7, and 8 also notably carried the c.1239A>C variation of *OPN*, associated with ALPS and DALD [36]. Further, patients 2 and 8 displayed hemizygosity for the -349T variation of *SH2D1A*, which has been associated with ALPS and DALD [37]. Finally, patient 7 also carried the p.Ala91Val variation of *PRF1*, which has been associated with DALD [22].

ALPS and DALD display a similar clinical picture and share an inherited defect of Fas function and the modifying effect exerted by variants of *OPN*, *PRF1*, and *SH2D1A*. However, DN T cell expansion is only present in ALPS, which may mark immunopathological differences because a direct role has been ascribed to these cells in ALPS development. This work shows that mutations of *UNC13D* may also represent an immunologic difference because they were detected in ALPS but not in DALD patients. Moreover, the *UNC13D* variations were not detected in MS patients, which suggested that they are not a common risk factor for autoimmunity. However, a possible role of *UNC13D* in development of autoimmune diseases other than ALPS has been previously suggested in patients with SJIA [30,38], who may also display decreased NK function [39]. By contrast, the *OPN* and *PRF1* variants were involved in development of several other autoimmune diseases [40–47]. Future whole-genome or exome sequencing studies will reveal the complex genetic scenario that may contribute to ALPS and DALD.

Author Contributions

Conceived and designed the experiments: MA SRE CD UR UD. Performed the experiments: VC EO ID SB MFS GC EB MM SM NC. Analyzed the data: DP AC. Contributed reagents/materials/analysis tools: UD UR. Wrote the manuscript: MA SRE CD UR UD. Recruitment and diagnosis of ALPS patients: SP UR. Recruitment and diagnosis of MS patients: CC.

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4. ARTICLE 2:

The -346T polymorphism of the SH2D1A gene is a risk factor for development of autoimmunity/lymphoproliferation in males with defective Fas function

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The -346T polymorphism of the *SH2D1A* gene is a risk factor for development of autoimmunity/lymphoproliferation in males with defective Fas function

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ABSTRACT

Inherited defects decreasing function of the Fas death receptor cause autoimmune lymphoproliferative syndrome (ALPS) and its variant Dianzani autoimmune lymphoproliferative disease (DALD). Since a deleterious mutation of the *SH2D1A* gene protects MRL*lpr/lpr* mice from ALPS development, we investigated the role of *SH2D1A*, located in the X chromosome, in 51 patients with ALPS or DALD by mutational screening of coding and regulative sequences. Allelic frequency of the -346C>T polymorphism was different in male patients and controls (-346T: 61% vs 36%, $p = 0.01$), with similar frequencies in ALPS and DALD. By contrast, no differences were found among females or between the controls and patients with multiple sclerosis (229 males, 157 females). Further analyses showed that -346C was a methylation site in $CD8^+$ T and natural killer cells, and *SH2D1A* expression was higher in -346T than in -346C males. Finally, *in vitro*–activated T cells from -346T males produced lower amounts of interferon-γ than those from -346C males. These data suggest that -346T is a predisposing factor for ALPS and DALD in males possibly because of its effect on SAP expression influencing the T-cell response.

1. Introduction

Fas/Apo-1 (CD95) is a ubiquitous death receptor, and cells expressing it undergo apoptosis upon interaction with Fas ligand (FasL) [1–3]. In lymphocytes, Fas triggering does not induce apoptosis in resting and recently activated T cells, but the apoptosis-inducing pathway is connected to Fas several days after cell activation. This Fas/FasL interaction is involved in shutting off immune responses, regulating the lymphocyte lifespan, and maintaining peripheral tolerance. Moreover, cytotoxic T lymphocytes (CTL), T helper 1 (T_{H1}), and natural killer (NK) cells express FasL, whose interaction with Fas expressed by target cells is one of the mechanisms that they use to exert their cytotoxic function.

The autoimmune lymphoproliferative syndrome (ALPS) is characterized by defective function of Fas, autoimmune manifestations that predominantly involve blood cells (*ie*, thrombocytopenia, anemia, neutropenia), polyclonal accumulation of lymphocytes in the spleen and lymph nodes, and peripheral expansion of T-cell receptor (TCR) $\alpha\beta^+$ CD4/CD8 double-negative (DN) T cells [4–9]. Moreover, ALPS patients are predisposed to develop lymphomas in adulthood [10].

Most ALPS cases are caused by deleterious mutations of the Fas gene (ALPS-FAS), although a few patients carry mutations of the FasL gene (ALPS-FASL) or the caspase 10 gene (ALPS-CASP10). However, the mutated gene is not known in a substantial proportion of patients (ALPS-U) [4–9]. In mice, a disease overlapping ALPS is displayed by MRL*lpr/lpr* and *gld/gld* mice, carrying mutations of the Fas and the FasL gene, respectively [1,2,11]. Moreover, we described an incomplete form of ALPS fulfilling the first three criteria but lacking an expansion of DN T cells. These patients did not display Fas, FasL, or caspase 10 mutations, although most of their parents displayed the Fas function defect, and we suggested an involvement of inherited mutations hitting the Fas pathway downstream from the receptor [9,12–14]. Because the complete paradigm of ALPS could not be demonstrated, this form has been named Dianzani autoimmune lymphoproliferative disease (DALD) by McKusick (OMIM Database ID: 601240, reference %605233) [9].

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expression assay (Assay-on Demand; SAP, Assay No. Hs00158978_m1; Applied Biosystems). The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH Assay No. Hs99999905_m1; Applied Biosystem) was used to normalize for cDNA amounts. Real-time PCR was performed using the 7000 Sequence Detection System (Applied Biosystems) in duplicate for each sample in a 20 μl final volume containing 1 μl diluted cDNA, 10 μl TaqMan universal PCR master mix (Applied Biosystems), and 1 μl Assay-on Demand mix. The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed with a Delta-Delta CT method.

2.6. Western blot analysis

Cells were washed twice with PBS and lysed for 20 min on ice in a lysis buffer (150 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 8, 0.5% Nonidet P40 Substitute, 5 mM EDTA, aprotinin 1 μg/ml, leupeptin 1 μg/ml, pepstatin A 1 μg/ml, PMSF 100 μg/ml). Lysates were then cleared by centrifugation for 20 min at 13,000 rpm at 4°C and separated on 12% SDS-PAGE gel after denaturation in SDS-PAGE loading buffer (63 mmol/l Tris-HCl pH 6.8, 5% glycerol, 1% SDS, 2.5% bromophenol-blue), and transferred to nitrocellulose. Filters were then blocked in TBST buffer plus 5% non-fat milk for 1 hour and then incubated with anti-SAP monoclonal antibody (mAb) (Upstate, Lake Placid, NY) overnight at 4°C in TBST buffer plus 5% bovine serum albumin. SAP signals were revealed with a peroxidase-conjugated anti-mouse Ig secondary antibody (GE Healthcare, Bockinghamshire, UK) and detected by enhanced chemiluminescence. β-Actin was detected with mouse mAb (Sigma) and detected with the same secondary antibody. Bands were quantified with the GelDoc EQ system (BioRad, Hercules, CA).

2.7. Functional assays

PBMC were cultured for 9 days in RPMI 1640 supplemented with 10% FBS, PHA (1 μg/ml) and IL-2 (2 U/ml) to increase SLAM expression. Then, cells ($1x10^5$/well) were seeded in round-bottom 96-well plates pre-coated with 100 μl of anti-CD3 mAb (OKT3, 1 μg/ml) overnight at 4°C, and cultured for 72 hours in the presence and absence of soluble anti-SLAM mAb (1 μg/ml; BioLegend, San Diego, CA). Secretion of cytokines was evaluated in the supernatants after 72 hours of culture by capture enzyme-linked immunosorbent assay (ELISA): Interleukin (IL)–4 and IFN-γ using ELISA MAX Deluxe (BioLegend), IL-10 and IL-17 using Human DuoSet (R&D Systems, Minneapolis, MN). To analyze cell proliferation, [^{3}H] thymidine (0.5 μCi/well) was added during the last 6 hours of culture; cells were then harvested and [^{3}H] thymidine uptake was evaluated with a β-counter (Perkin Elmer, Norwalk, CT).

2.8. Statistical analysis

Statistical analyses of allele and genotype distributions were performed using the χ^2 test with the Yates correction. Haplotype analysis was performed using the Haploview program (3.11 version, Broad Institute of MIT and Harvard, 2003-2006). This program also calculated pairwise linkage disequilibrium (LD) values (r^2 and Lewontin D′) among the SNP alleles. Allele transmission from the mothers to the affected sons was evaluated according to the Affected Family-BAsed Control (AFBAC) method. In families ascertained for the presence of an affected child, the parental alleles not transmitted to the affected child were used as "control" alleles, whereas the parental transmitted alleles were used as "case" alleles. Transmission Disequilibrium Test (TDT) analysis was performed using Haploview program. The use of nuclear family data in case-control association studies was developed to avoid possible ethnic mismatching between patients and randomly ascertained controls. Functional data were analyzed with the nonparametric Mann–Whitney *U* test.

3. Results

3.1. Genetic analysis

To assess the role of *SH2D1A* in development of ALPS and DALD, the genomic DNA corresponding to the 5′UTR, the four exons and their intron/exon boundaries, and the 3′UTR of *SH2D1A* were sequenced in 31 male patients with ALPS or DALD (6 ALPS-FAS, 9 ALPS-U, and 16 DALD), and 369 male healthy controls, as variations of a X-linked gene would have a prominent effect in males. In both patients and controls, we only found three SNPs, *ie*, -346C>T (rs12164382) in the 5′UTR, and -494G>A (rs7357894) and -631G>A (rs990545) in the 5′ flanking region (numeration is referred to ATG = +1). Results showed that the frequencies of the -346T and -631A alleles were significantly higher in the patients than in the controls (-346T: 61% vs 36%; -631A: 61% vs 37%) and conferred an OR of 2.78 (95% CI = 1.24–6.30; $p = 0.01$) and 2.74 (95% CI = 1.22–6.22; $p = 0.01$), respectively, for ALPS or DALD development (Table 1). No significant difference was found in the frequency of -494G>A.

To assess whether a similar association was detectable in females too, we typed the three SNPs in 20 female patients with ALPS or DALD (three ALPS-FAS, nine ALPS-U, eight DALD) and 165 matched controls. Results showed that allelic and genotypic frequencies were similar in patients and controls among the females. Moreover, allelic frequency of -346T and -631A were significantly higher in the males than in the females among the patients (-346T: 61% vs 31%, $p = 0.029$; -631A: 61% vs 34%, $p = 0.049$). No significant difference was found in the frequency of the -494G>A alleles (Table 2).

Highly significant pairwise LD was observed between the three SNPs and, in particular, -346C>T was in almost perfect LD with -631G>A (D′ 0.96, $r^2 = 0.8$) (Table 3).

The Transcription Element Search System analysis program (http://www.cbil.upenn.edu/cgi-bin/tess) showed that the three SNPs did not modify the putative binding sites of transcription factors, but -346C was reported as a putative methylation site [25], suggesting that -346C>T may influence the gene expression. Therefore, we focused our further analyses on -346C>T.

Table 1
Allele frequencies of different SNPs in *SH2D1A* in male ALPS/DALD patients and healthy controls

-346C>T			-494G>A			-631G>A		
Allele	Controls[a]	Patients	Allele	Controls	Patients	Allele	Controls	Patients
C	235 (64%)	12 (39%)	G	215 (58%)	22 (71%)	G	234 (63%)	12 (39%)
T	134 (36%)	19 (61%)	A	154 (42%)	9 (29%)	A	135 (37%)	19 (61%)
Total	369	31		369	31		369	31
OR = 2.78 (95% CI = 1.24–6.30) $p = 0.01$[b]	NS $p = 0.01$	OR = 2.74 (95% CI = 1.22–6.22)						

CI, confidence interval; NS, Not significant; OR, odds ratio.
[a]Number of subjects; percentages are shown in parentheses.
[b]Patients vs controls; p value uncorrected for number of comparisons.

Table 2
Allele frequencies of different SNPs in *SH2D1A* in female ALPS/DALD patients and healthy controls

-346C>T			-494G>A			-631G>A		
Allele	Controls[a]	Patients	Allele	Controls	Patients	Allele	Controls	Patients
C	206 (62%)	27 (69%)	G	191 (58%)	25 (59%)	G	203 (61%)	26 (66%)
T	124 (38%)	13 (31%)	A	139 (42%)	15 (41%)	A	127 (38%)	14 (34%)
Total	330	40		330	40		330	40
Genotype	Controls[a]	Patients	Genotype	Controls	Patients	Genotype	Controls	Patients
CC	67[b] (40%)	9 (45%)	GG	55 (33%)	8 (40%)	GG	65 (40%)	9 (45%)
CT	72 (44%)	9 (45%)	GA	81 (49%)	9 (45%)	GA	73 (44%)	8 (40%)
TT	26 (16%)	2 (10%)	AA	29 (18%)	3 (15%)	AA	27 (16%)	3 (15%)
Total	165	20		165	20		165	20

[a]Number of chromosomes; percentages are shown in parentheses.
[b]Number of subjects; percentages are shown in parentheses.

Dissection of data according to the ALPS or DALD diagnosis showed that frequency of -346T was almost identical in ALPS and DALD patients in both males (ALPS: 60%, n = 15; DALD: 62%, n = 16) and females (ALPS: 33%, n = 12; DALD: 31%, n = 8). Clinical analysis did not detect any substantial difference between -346T and -346C patients in terms of the lymphoproliferative picture and autoimmune manifestations (data not shown).

Typing of the -346C>T alleles in the available fathers (n = 21) and mothers (n = 22) of the male patients showed that, among the non transmitted alleles (paternal alleles and non transmitted maternal alleles), frequencies (-346C: 60%; -346T: 40%) were similar to those detected in the healthy control groups, which showed that the general population used as a control was ethnically matched with the patients' families. Intriguingly, analysis of the heterozygous mothers by the TDT analysis showed a preferential transmission of -346T (transmitted:non transmitted alleles, 9:4), but results did not reach statistical significance because of the small number of subjects.

To assess whether -346T has a predisposing effect on autoimmune diseases different from ALPS and DALD, we typed this SNP in 386 patients with MS (229 males and 157 females). Results showed that allelic frequency of -346T was similar in MS patients (38% in both males and females) and the controls, with no gender differences (data not shown).

3.2. Functional analyses

To investigate whether the -346C>T alleles influence SAP expression, we analyzed the SAP mRNA level by real-Time PCR in PBMC after 3 days of culture in the presence of PHA and IL-2, as preliminary experiments showed that SAP was minimally expressed in fresh cells but was substantially upregulated in these culture conditions. This analysis was performed in PBMC from males carrying -346C (healthy controls n = 10; patients n = 5) or -346T (healthy controls n = 10; patients n = 4). Results showed that -346T carriers, either controls or patients, displayed higher levels of SAP mRNA than the respective -346C carriers ($p = 0.001$ and $p = 0.01$, respectively) (Fig. 1). In some experiments, the cultured PBMC from the healthy controls (-346C: n = 6; -346T: n = 6) were used to positively purify the $CD4^+$ (T_H), $CD8^+$ (CTL) and $CD16^+$ (NK) cell subsets using magnetic microbeads. Results showed that -346T carriers displayed significantly higher levels of SAP mRNA than -346C carriers, in CTL ($p = 0.009$) and NK ($p = 0.002$) cells; by contrast, the difference was not significant in T_H cells (Fig. 2, left panel). To rule out the possibility that these results were influenced by the positive purification technique, CTL and NK cells (-346C: n = 5; -346T: n = 5) were negatively purified from fresh PBMC using the RosetteSep technique and then cultured as reported above. Analysis of SAP expression confirmed that -346T carriers displayed higher levels of SAP mRNA than -346C carriers in both cell types (CTL: $p = 0.03$; NK: $p = 0.04$) (Figure 2, right panel). Moreover, the -346C samples were used to evaluate the methylation state of this site by methylation-specific PCR. Results showed that -346C was methylated and its methylation level was higher in PBMC than in CTL and NK cells (Figure 3). Methylation analysis of PBMC derived from -346C male patients (n = 5) confirmed methylation of this site (range 17%–29 %).

To assess whether the differential expression of SAP was detectable at the protein level too, we analyzed SAP expression by Western blot in PBMC from healthy males carrying -346C (n = 10) or -346T (n = 10), after 5 days of culture in the presence of PHA and IL-2. Results showed that -346T carriers displayed significantly higher levels of SAP than -346C carriers ($p = 0.02$) (Figure 4).

Table 3
Linkage disequilibrium pattern of the three SNPs

-631G>A	-494G>A	-346C>T
-631G>A	$D' = 1$ $r^2 = 0.5$	$D' = 0.96$ $r^2 = 0.8$
-494G>A	$D' = 1$ $r^2 = 0.5$	$D' = 0.95$ $r^2 = 0.46$
-346C>T	$D' = 0.96$ $r^2 = 0.8$	$D' = 0.95$ $r^2 = 0.46$

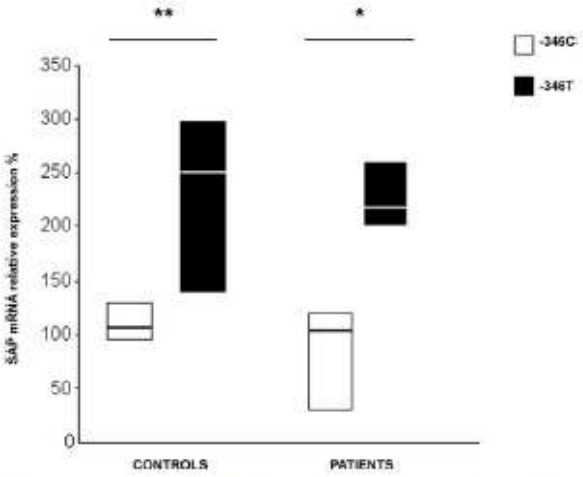

Fig. 1. SAP expression at the mRNA level in activated PBMC derived from healthy males and patients carrying -346C or -346T. PBMC from male patients (-346C: n = 5, -346T: n = 4) and healthy controls (-346C: n = 10, -346T: n = 10) were cultured in the presence of PHA and IL-2 for 3 days. Quantification of the SAP mRNA was evaluated by real-time PCR. The median expression level detected in -346C samples in each experiment was 100% expression. Horizontal lines show the median values and boxes the interquartile ranges. Statistical analyses were performed with the non parametric Mann–Whitney *U* test (**$p < 0.01$; *$p < 0.05$).

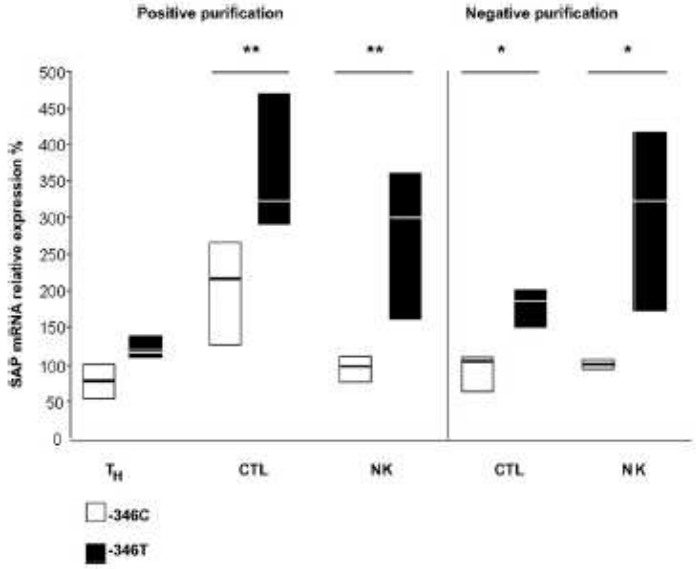

Fig. 2. SAP expression at the mRNA level in T_H, CTL, and NK cells derived from healthy males carrying -346C or -346T by positive and negative purification. Cell subsets were positively purified from PHA-activated PBMC (-346C: n = 6, -346T: n = 6) by magnetic microbeads (left panel) or negatively purified from fresh resting PBMC (-346C: n = 5, -346T: n = 5) and then cultured in the presence of PHA and IL-2 for 3 days (right panel). Quantification of the amount of the SAP mRNA was evaluated by real-time PCR and data are expressed as in Figure 1.

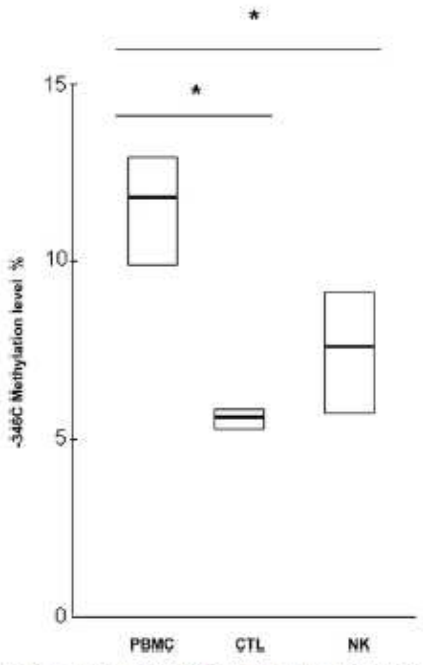

Fig. 3. SAP -346C methylation in PBMC, CTL, and NK cells derived from healthy males. Quantitative analysis of DNA methylation at the -346C site in amplified bisulphite CT-converted genomic DNA from the cells obtained from the -346C healthy males as described for the right panel of Figure 2. The methylation level is expressed as median values and interquartile ranges of the relative proportion of the unconverted methylated C and the T-converted unmethylated C. Statistical analyses were performed with the non parametric Mann–Whitney *U* test (*$p < 0.05$).

To assess whether the -346C>T variants influenced T-cell activation, we analyzed proliferation and cytokine secretion in T cells from healthy males carrying -346C (n = 8) or -346T (n = 8). PBMC were cultured for 9 days with PHA and IL-2 to increase SLAM expression. They were then activated with anti-CD3 mAb in the presence and absence of anti-SLAM mAb, and secretion of IFN-γ, IL-4, IL-10, and IL-17, and cell proliferation were evaluated after 3 days. Results showed that -346T carriers produced significantly less IFN-γ than -346C carriers without substantial differences in the presence and absence of SLAM stimulation (Figure 5, left panel). By contrast, no substantial differences were found in secretion of IL-4, IL-10, and IL-17 (data not shown), and cell proliferation (Figure 5, right panel) between -346C and -346T carriers. These experiments also showed that SLAM stimulation similarly increased cell proliferation in -346C and -346T carriers, but it did not modulate cytokine secretion.

4. Discussion

This work shows that the -346C>T variation of *SH2D1A* is associated with ALPS and DALD and hits a methylation site involved in SAP expression. In particular, patients with ALPS and DALD showed an increased frequency of the -346T allele that lacked the -346C methylation site and was associated with increased expression of SAP in NK and $CD8^+$ T cells and decreased secretion of IFN-γ. These data support the possibility of an opposite epistatic relationship between Fas and SAP expression, which has been previously suggested in MRL*lpr/lpr* mice in which a spontaneous A insertion at the 21st codon of *SH2D1A* (first exon) causing a frame-shift had resulted in defective expression of SAP and a striking reduction of hypergammaglobulinemia, autoantibody production, DN T-cells counts, lymphadenopathy, splenomegaly, and pathological indexes for glomerulonephritis and vasculitis [24].

In our patients and controls, we detected three SNP that were in strong LD and, in particular, -346C>T and -631G>A, which were significantly associated with ALPS/DALD, were in almost perfect LD, whereas their LD with -494G>A was weaker. The -346C>T SNP was the only one with a likely functional effect. The three SNPs

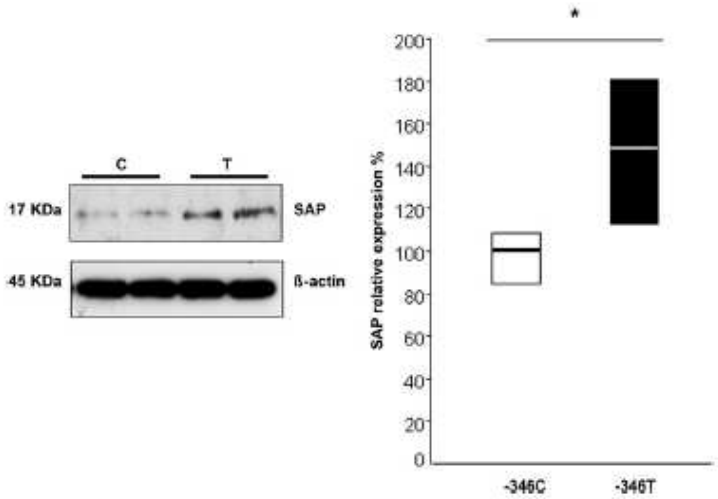

Fig. 4. SAP expression at the protein level in PBMC derived from healthy males carrying -346C or -346T. PBMC from healthy males (-346C: n = 10, -346T: n = 10) were cultured in the presence of PHA and IL-2 for 5 days and then SAP expression was evaluated by western blot. Left panel: Representative western blots of two -346 C and two -346 T samples. (Right panel) Densitometric quantification of SAP of results from all experiments. The median expression detected in the -346C samples in each experiment was 100% expression. Horizontal lines show the median values and boxes the interquartile ranges; statistical analysis was performed with the Mann–Whitney *U* test, (*$p < 0.05$).

were not included in transcription factor binding sites, but -346C had previously been shown to be a putative methylation site [25], and our work experimentally confirmed this possibility and showed that -346T hemizygotes expressed higher levels of SAP than -346C hemizygotes in NK and $CD8^+$ T cells. Therefore, our data strongly suggest that -346T is directly responsible for the predisposing effect to ALPS/DALD development by increasing SAP expression. The finding that association with -346T was detected only in males and that, among patients, males displayed significantly higher frequency of -346T than females may reflect differential use of the -346C methylation site or other regulatory sites in males and females. This would be intriguing, as a gender influence in the ALPS picture was previously reported by Maric et al. [26], who showed that lymph nodes from male ALPS patients frequently displayed histopathological features of sinus histiocytosis with massive lymphadenopathy, that were rare in female ALPS patients.

SAP has two functional domains for protein interaction [23,27–31]. The first is a SH2 domain which binds to a unique class of cytoplasmic tyrosine-based motifs present in receptors belonging to the SLAM family; SAP binds this site with high affinity and blocks its interaction with the tyrosine phosphatase SHP-2. The second domain binds the tyrosine kinase Fyn that propagates downstream signals that are essential to the function of the SLAM family members. These signals may play an important role to develop autoimmunity and lymphadenopathy in MRL*lpr/lpr* mice since *lpr* T cells show an unusual activation of Fyn, that may support their survival [32,33]. Moreover, Fyn deficiency resulted in a marked reduction of lymphadenopathy and autoantibody production in these mice [34], that mimicked the effect of SAP deficiency.

ALPS and DALD share the clinical picture and defective Fas function, but are distinguished by DN T-cell expansion that is only present in ALPS. This difference is important from a diagnostic

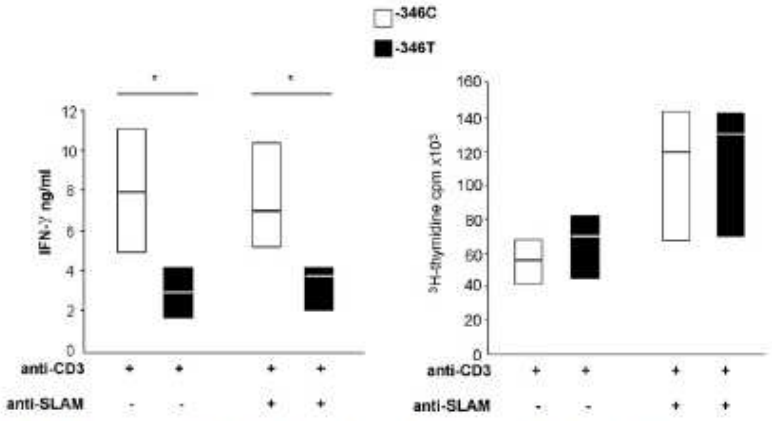

Fig. 5. IFN-γ production and proliferation in T cells from healthy males carrying -346C or -346T. PBMC from healthy controls (-346C: n = 8, -346T: n = 8) were cultured for 9 days in the presence of PHA and IL-2, then restimulated with anti-CD3 mAb for 3 days in the presence and absence of anti-SLAM mAb. Then, IFN-γ secretion (left panel) and cell proliferation (right panel) were measured. Horizontal lines show the median values and boxes the interquartile ranges. Statistical analysis was performed with the Mann–Whitney *U* test, (*$p < 0.05$).

point of view since the search for DN T cells is a first level laboratory analysis to diagnose ALPS, but it may also mark immunopathologic differences as DN T cells might play a direct role in ALPS development. Despite these differences, this work on SAP and our previous works on the *OPN* and *PRF* genes indicated that the genetic background predisposing to ALPS and DALD may be similar, as variations of these three genes predispose to both diseases. We previously suggested that the *OPN* and *PRF* gene variations cooperated with the Fas defect in slowing down the immune response switching off by inhibiting AICD and cytotoxic cell-mediated fratricide of activated immune cells, respectively, that flank Fas-induced apoptosis in eliminating activated immune cells [16–18]. A similar mechanism might also be involved in the predisposing effect mediated by -346T, as this variation was associated with decreased secretion of IFN-γ, that is a cytokine able to enhance FasL expression in T_H cells [35]. By contrast, the effect was not due to a direct effect on AICD and Fas-induced cell death, as they were similar in T cells derived from healthy males carrying -346C or -346T (data not shown). It is noteworthy that this picture is different from that displayed by XLP patients showing defective AICD and normal Fas-induced apoptosis [36].

The SLAM family of receptors comprise seven members: SLAM (CD150), 2B4 (CD244), CD84, CD48, NTBA (SLAMF6 or Ly108 in the mouse), Ly9 (CD229), and CD2-like receptor activating cytotoxic cells (CRACC or CD319) [27,30,31]. Most SLAM family members form homophilic interactions, whereas 2B4, an activating receptor for NK cells and $CD8^+$ cytotoxic T cells, interacts with CD48 upregulated by EBV-infected B cells; the abnormal responses to EBV infection displayed by XLP patients are probably due in part to defects of 2B4-mediated cytotoxicity. However, 2B4 seemed not to play a role in our system since 2B4-mediated cytotoxicity was similar in -346C and -346T carriers (data not shown). Moreover, no role was found for SLAM whose triggering did not influence the differential IFN-γ secretion displayed by these donors.

A general role of SAP in autoimmunity has been suggested by the finding that SAP-deficient mice are protected not only from the *lpr* disease but also from EAE and pristane-induced lupus [31]. However, this general role does not seem to involve -346C>T, as we did not detect any association of this variation with MS. This makes a difference with the osteopontin and perforin gene variations that were found to be associated not only with ALPS and DALD but also with MS and other autoimmune diseases [37–40].

In conclusion, this work suggests that high SAP expression may favor lymphoproliferation in ALPS and DALD patients, and opens the way to the possibility that partial pharmacological inhibition of SAP might be beneficial to control these diseases.

Acknowledgments

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5. ARTICLE 3:

IL-17 protects T cells from apoptosis and contributes to development of ALPS-like phenotypes

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IL-17 protects T cells from apoptosis and contributes to development of ALPS-like phenotypes

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IL-17 protects T cells from apoptosis and contributes to development of ALPS-like phenotypes.

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Key Points

In vitro, IL-17 inhibits Fas-induced cell death and IL-17 neutralization improves lymphocyte apoptosis in patients with ALPS and DALD.

Treatment of MRL*lpr/lpr* mice with anti-IL-17A antibodies decreases the severity of autoimmune/lymphoproliferative disease.

Abstract

In autoimmune/lymphoproliferative syndrome (ALPS), defective Fas death receptor function causes lymphadenomegaly/splenomegaly, the expansion of TCR$\alpha\beta^+$ CD4/CD8 double-negative (DN) T cells, and frequent development of hematologic autoimmunity. Dianzani Autoimmune Lymphoproliferative Disease (DALD) has a similar phenotype but lacks the expansion of DN T cells. This work shows that patients with ALPS and DALD have high serum levels of IL-17A, IL-17F and IL-17AF, which are involved in several autoimmune diseases, and that their T cells show increased secretion of these cytokines upon activation in vitro. The following data indicate that these cytokines may contribute to ALPS and DALD: 1) recombinant IL-17A and IL-17F significantly inhibit Fas-induced cell death (FICD) in Fas-sensitive T cells from healthy donors; 2) this inhibitory effect is also induced by the patients' serum and is reversed by anti-IL-17A antibodies; 3) IL-17A neutralization substantially increases FICD in T cells from ALPS and DALD patients in vitro and 4) treatment with anti-IL-17A antibodies ameliorates the autoimmune manifestations and, at a lesser extent, the lymphoproliferative phenotype and prolongs survival in MRL*lpr/lpr* mice, which are an animal model of ALPS. These data suggest that IL-17A and IL-17F could be targeted therapeutically to improve Fas function in ALPS and DALD.

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a human genetic disorder of lymphocyte apoptosis resulting in the accumulation of polyclonal lymphocytes in the lymph nodes and in the spleen with expansion of T cell receptor αβ (TCRαβ)-positive CD4/CD8 double-negative (DN) T cells and frequent development of autoimmune manifestations, mainly hemocytopenias. The disease is caused by genetic mutations that affect Fas-mediated apoptosis and that have an autosomal dominant inheritance pattern with incomplete penetrance[1,2]. Most patients carry a heterozygous mutation in the *FAS* gene (ALPS-FAS), whereas very few patients carry mutations in the *FAS LIGAND* (ALPS-FASLG) or *CASPASE10* (ALPS-CASP10) genes; however, the causal mutation is not known in a substantial proportion of patients (ALPS-UND). Moreover, a substantial number of patients carry somatic mutations in *FAS* in the DN T cell population (ALPS-sFAS)[3]. We have also described patients with lymphadenomegaly/splenomegaly, autoimmune manifestations and defective Fas function, but without expansion of DN T cells. This disease has been named Dianzani Autoimmune Lymphoproliferative Disease (DALD) (OMIM reference #605233)[3], and several features indicate that it may involve genes in the Fas pathway[4,5].

In previous work, we showed that ALPS and DALD patients have high serum levels of Osteopontin (OPN)[6] and Tissue Inhibitor of Metalloproteinase-1 (TIMP-1)[7]. An increase in OPN was partly due to polymorphisms in its gene and might cause the observed increase in TIMP-1 because OPN induces TIMP-1 secretion in monocytes. Because both OPN and TIMP-1 inhibit lymphocyte apoptosis in vitro, we speculated that their high levels may contribute to ALPS and DALD development in subjects with a hypofunctional Fas system[6,7]. Moreover, the role of risk factor may be also played by gene variations decreasing function of perforin, involved in the function of cytotoxic T cells and natural killer cells[8-10].

OPN is a proinflammatory cytokine that is also involved in the development of T helper 17 (Th17) cells[11]. These are proinflammatory T helper (Th) cells characterized by the secretion of IL-17A and

IL-17F, which can be secreted either as homodimers or as IL-17AF heterodimers[12]. IL-17A and IL-17F can be also produced by other cell types, including $CD8^+$ T cells, γδ T cells, natural killer cells and neutrophils[13]. They share about 50% sequence identity and several biological activities, including neutrophil recruitment and induction of proinflammatory cytokines, chemokines, and metalloproteinases[14]. However, their receptor usage is partly different, their secretion is independently regulated in Th cells, they display different proinflammatory activity, and are differently expressed in several autoimmune and allergic diseases[15-17].

Increasing evidence indicates that Th17 cells play an important role in several autoimmune diseases, including multiple sclerosis (MS), psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease, anti-neutrophil cytoplasmic antibodies associated vasculitis, and systemic lupus erythematosus (SLE)[18-22]. SLE patients, in particular, have high serum levels of IL-17A and IL-23 and increased numbers of IL-17-producing T cells in the peripheral blood (PB), and these high levels correlate with disease activity. Moreover, in vitro experiments have shown that IL-17A increases autoantibody production (anti-dsDNA, anti-nuclear) in the peripheral blood mononuclear cells (PBMCs) of patients with lupus nephritis, protects B cells from activation-induced cell death (AICD), and supports their proliferation and differentiation into immunoglobulin (Ig)-secreting cells[23-26].

Intriguingly, some SLE patients show expansion of DN T cell populations, a hallmark of ALPS, and secrete high levels of IL-17A. Moreover, high levels of IL-17A are produced by DN T cells that infiltrate the nephritic kidneys in MRL*lpr/lpr* mice. These are used as an animal model of ALPS because they carry mutations in the *FAS* gene and have a typical ALPS-like phenotype with lymphadenomegaly/splenomegaly and expansion of DN T cells, but they also have features that are atypical of ALPS patients, such as lupus-like nephritis and anti-dsDNA autoantibodies[25,26].

These observations prompted the present work, in which we investigate the roles of IL-17A and IL-17F in ALPS and DALD. Our results show that the levels of IL-17A, IL-17F, and IL-17AF are

increased in ALPS and DALD patients and that IL-17A and IL-17F inhibit Fas-induced cell death (FICD) in vitro. Moreover, treatment of MRL*lpr/lpr* mice with anti-IL-17A antibodies decreases the severity of autoimmune/lymphoproliferative disease, decreases renal involvement, and prolongs survival.

Methods

Patients

We analyzed 18 ALPS (n=9 ALPS-FAS; n=1 ALPS-sFAS; and n=8 ALPS-UND) and 18 DALD patients who were followed at the Pediatric Department, University of Turin, Italy, and age-matched healthy controls (n=50). ALPS and DALD were diagnosed according to the criteria indicated in the 2009 ALPS NIH International Workshop[3]. Clinical and laboratory data are reported in the Supplemental Table 1. Most patients had received corticosteroid therapy but analyses were always performed at least four weeks from the last treatment.

Written informed consent was obtained from patients and controls. This study was conducted in accordance with the Declaration of Helsinki. The study was planned according to the guidelines of the local ethical committee.

Cytokine secretion

Cytokine serum levels were evaluated by (Enzyme-linked immunosorbent assay) ELISA (R&D system Minneapolis, USA; eBioscience, San Diego, CA, USA).

PBMCs ($1x10^5$) were cultured for 5 days in round-bottomed 96-well plates in the presence of anti-CD3 (10 µg/mL) and an anti-CD28 (1 µg/mL; Ancell, Bayport, USA) monoclonal antibody (mAb) and in the presence of recombinant (r) IL-23 (rIL-23 50 ng/mL; R&D system). Then, IL-17A and IL17F were measured by ELISA on the supernatant; absorbance was evaluated using a microplate reader (Bio-Rad, Hercules, CA, USA), and the I-smart program was used to calculate the standard curve. Cells were stained with a PE-conjugated anti-CCR6 mAb and an APC-conjugated anti-CD4 mAb (eBioscience). Alternatively, they were re-stimulated with Phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma, Saint Louis, MO) plus Ionomycin (500 ng/mL; Sigma) for 5 hours in the presence of Brefeldin-A (10 µg/mL; Sigma), permeabilized, and stained with a FITC-conjugated

anti-TCR$\alpha\beta^+$ mAb and an Alexa Fluo 647-conjugated anti-IL-17A mAb (eBioscience). Then, they were analyzed by flow cytometry.

FICD assay

PBMCs were separated by density-gradient centrifugation. FICD was evaluated as previously reported[4,5] in activated PBMCs using a soluble anti-Fas mAb (0.5 µg/mL CH11; UPSTATE Waltham, MA, USA) in the presence and absence of rIL-17A or rIL-17F (5 ng/mL; Peprotech, Rocky Hill, USA). In some experiments, FICD was also induced in the presence of a neutralizing anti-IL-17A antibody (10 µg/mL; R&D system). After 16 hours, cell survival was assessed by counting the live cells in each well using the trypan blue exclusion test. The results are expressed as relative cell survival % calculated as follows: (total live cell count in the assay well/total live cell count in the respective control well) × 100.

In some experiments, cells were harvested at the end of the FIDC assay and lysed in 150 mM NaCl, 20 mM Tris-HCl–pH 8, 0.5% Nonidet P40 Substitute, 5 mM EDTA, aprotinin 1 µg/mL, leupeptin 1 µg/mL, pepstatin A 1 µg/mL, PMSF 100 µg/ml for 30 min. Lysates were then separated by SDS-PAGE, transferred to Hybond-C extra membranes (Ge Healthcare, Piscataway, NJ, USA), blotted with antibodies to FLIP (Alexis Axxora, San Diego, CA), XIAP (Alexis), Bcl-2 (Stressgen, Victoria, BC, Canada), and β-actin (Sigma) and a peroxidase-conjugated anti-mouse Ig secondary antibody (Ge Healthcare), and revealed by chemiluminescence.

Mice

Female MRL*lpr/lpr* mice (stock no. 000485) were purchased from The Jackson Laboratory. Eight-week-old MRL*lpr/lpr* females were randomized into 3 groups of 8 mice each and were treated with 4 intraperitoneal injections (one every 4 days) of PBS, anti-IL-17A antibody (100 µg/100 µL, R&D System), or an isotype-matched control ($IgG2_A$, R&D System). The concentration of anti-dsDNA

antibodies was evaluated by ELISA (Alpha Diagnostic International, San Antonio, USA) in mouse sera (Low NSB Sample Diluent) at a 1:500 dilution. Proteinuria was evaluated using reagent strips (A. Menarini Diagnostic, Berkshire, UK) and was ranked as follows: 0 (negative); Pro.1+ (30 mg/dL); Pro.2+ (100 mg/dL); Pro.3+ (300 mg/dL); and Pro.4+ (1000 mg/dL). The lymph node and spleen sizes were expressed as the ratio between the organ wet weight (g) and the body weight (g) x 100. The experiments were approved by the local ethical committee for animal experimentation.

Statistical analysis

The Mann-Whitney U-test was used to compare unpaired data from different groups, Wilcoxon's signed rank test was used for the analysis of paired data, and Fisher's exact test was used for the comparison of high IL-17 groups.

A Kaplan–Meier survival analysis with a log-rank test (Mantel-Cox) of the statistics was used to assess the survival rate and to compare the differences between the survival curves. All P values are 2-tailed, and the significance cut-off is $P < .05$. The statistical analyses were performed with GraphPad Instat software (GraphPad Software, San Diego, CA, USA).

Results

Increased serum levels of IL-17A, IL-17F, and IL-17AF in ALPS and DALD patients

We measured IL-17A, IL-17F, and IL-17AF (IL-17s) by ELISA in the sera of 18 ALPS and 18 DALD patients and of 50 healthy matched controls. The levels of both IL-17A (ALPS: median 60 pg/mL, *Interquartile Range*, IQR 37-221; DALD: 44 pg/mL, 37-229) and IL-17F (ALPS: 59 pg/mL, 37-156; DALD: 61 pg/mL, 37-172) were significantly higher in the ALPS and DALD patients than in the controls (IL-17A: 37 pg/mL, 22-93; IL-17F: 12 pg/mL, 4-46) (Figure 1), whereas the levels of IL-17AF were significantly higher in the ALPS (346 pg/mL, 298-514) but not in the DALD (140 pg/mL, 30-389) patients than in the controls (56 pg/mL, 19-311) (Figure 1). By setting the thresholds at the 75th (93 pg/mL) and 95th (286 pg/mL) percentiles of the control values, single patient analysis showed that the levels of at least one of these cytokines were over the 75th percentile in 83% of the ALPS ($P < .0001$ vs. controls, Fisher exact test) and 56% of the DALD patients ($P = .002$) and greater than the 95th percentile in 33% of the ALPS and 33% of the DALD patients ($P = .02$).

Then, we compared the serum levels of IL-17s with those of IL-10 and IL-18, which are frequently elevated in ALPS patients, and IL-1β, known to support production of IL-17s. As shown in Figure 2 and supplemental Table 1, IL-18 levels were higher in the ALPS (1262 pg/mL, 337-2356) patients than in the DALD (113 pg/mL, 54-381) patients and the controls (181 pg/mL, 134-252). IL-10 levels were higher in the ALPS (46 pg/mL, 10-187) patients but not in the DALD (11 pg/mL, 7-24) patients than in the controls (10 pg/mL, 2-64). IL-1β levels were higher in the ALPS (27 pg/mL, 7-94) and the DALD (78 pg/mL, 11-192) patients than in the controls (6 pg/mL, 3-18). However, no significant correlation was found between the levels of these cytokines and the levels of IL-17s (data not shown).

PB Th17 cells are increased in ALPS and DALD patients

To assess whether Th17 cells are involved in the high serum levels of IL-17s, we evaluated IL-17A and IL-17F secretion by PBMCs from 15 patients (8 ALPS and 7 DALD) and 15 controls. PBMCs were activated by triggering CD3 + CD28 and were cultured for 5 days in the presence of rIL-23 to support Th17 cell expansion. At day 5, the proportions of $CD4^+CCR6^+$ cells and $TCR\alpha\beta^+IL\text{-}17A^+$ cells, comprising Th17 cells, were assessed by flow cytometry, and IL-17A and IL-17F secretion was assessed by ELISA in the supernatants.

The flow cytometry analysis showed that the proportions of $CD4^+CCR6^+$ and $TCR\alpha\beta^+IL\text{-}17A^+$ cells were higher in both patient groups than in the controls (mean±*Standard Error,* SE, $CD4^+CCR6^+$: ALPS 4.6%±1.3%, DALD 3.1%±1%, and controls 1.4% ± 0.3%; $TCR\alpha\beta^+IL\text{-}17A^+$: ALPS 1.66% ± 0.65%, DALD 3.5%±1.73%, and controls 0.65% ± 0.15%) (Figure 3A,B).

The ELISA evaluations showed that IL-17A secretion was significantly higher in the cultures from both of the patient groups than in those from the healthy controls (mean ± SE: ALPS 905 ± 305 pg/mL, DALD 944 ± 344, controls 295 ± 97). In contrast, IL-17F secretion was higher in the DALD patients than in the controls, but not in the ALPS patients (ALPS 853 ± 662 pg/mL, DALD 2979 ± 770, controls 843 ± 146) (Figure 3C,D).

rIL-17A and rIL-17F inhibit T cell apoptosis

Because ALPS and DALD are caused by defective Fas-induced lymphocyte apoptosis, we investigated the effects of IL-17A and IL-17F on FICD in Fas-sensitive T cells obtained from the healthy controls (n=7). The cells were treated with an anti-Fas mAb in the presence or absence of rIL-17A or rIL-17F and, after 16 hours, whole cell survival was assessed by the trypan blue exclusion test. The results show that both rIL-17A and rIL-17F significantly inhibited FICD, as detected by an increase in whole cell survival (Figure 4A), whereas these cytokines had no significant effect on the survival of cells not treated with the anti-Fas mAb. Moreover, titration

experiments using rIL-17A and rIL-17F in the 2.5 - 0.1 ng/mL concentration range showed that both cytokines protected T cells from FICD even at the lowest dose, which is comparable to the doses detected in the patients' sera (data not shown).

To investigate whether the anti-apoptotic effect of IL-17s was mediated by modulation of cellular inhibitors of FICD, we evaluated expression of cFLIP, XIAP and Bcl-2 at the end of the FICD assay. Western blotting with anti-FLIP antibodies detected two bands compatible with the isoforms $cFLIP_L$ and $cFLIP_S$ and showed that, in the absence of Fas stimulation, cells express high levels of $cFLIP_L$ whereas $cFLIP_S$ is undetectable; this pattern is not influenced by addition of IL-17A. By contrast, after Fas stimulation, cells express intermediate levels of both $cFLIP_S$ and $cFLIP_L$ in basal conditions, whereas, in the presence of IL-17A, they express high levels of $cFLIP_S$ but $cFLIP_L$ is undetectable (Figure 4B). By contrast, no effect was found in XIAP and Bcl-2 expression (not shown).

Neutralization of IL-17A in the sera of ALPS and DALD patients partly rescues lymphocytes' death

The titration experiments suggest that the IL-17A and IL-17F concentrations detected in the ALPS and DALD patients may contribute to the apoptotic defect in their lymphocytes. To confirm this possibility, we performed the FICD assay on Fas-sensitive T cells from healthy donors (n=9) in the presence of either FBS or serum from patient ALPS-5 (ALPS-sFAS), with high levels of IL-17A, but not of IL-17F, and in the presence or absence of anti-IL-17A neutralizing antibodies. The results show that FICD was induced at significantly higher levels in FBS than in the patient's serum. Moreover, the addition of anti-IL-17A antibodies had no substantial effect on FICD induced in the FBS-supplemented cultures, whereas it substantially increased the FICD induced in the patient's serum (Figure 5A). These results suggest that the IL-17A in the ALPS patient serum can inhibit FICD in Fas-sensitive T cells.

We then repeated these experiments in the T cells from 5 patients with ALPS (2 ALPS-FAS, 1 ALPS-sFAS and 2 ALPS-UND) and 4 with DALD by performing the FICD assay in either FBS or the autologous serum (AS) in the presence or absence of anti-IL-17A antibodies. Figure 5B shows that FICD was significantly higher in the presence of FBS than in the presence of AS. Moreover, the addition of the anti-IL-17A antibodies significantly increased FICD both in the presence of FBS and in the presence of AS. These data confirm that IL-17A contained in the serum of ALPS and DALD patients can further inhibit FICD in Fas-resistant cells and show that an inhibitory effect may also be exerted by the IL-17A endogenously secreted in vitro by the patients' cells in the FICD assay.

Effects of passive immunization with anti-IL-17A antibodies in MLR*lpr/lpr* mice

These results show that high levels of IL-17A and IL-17F may contribute to the apoptotic defect in the ALPS and DALD patients, and that neutralization of these cytokines may partly overcome the Fas function defect. To assess the role of IL-17A in vivo, we evaluated the effect of treatment with anti-IL-17A antibodies in the ALPS-like disease developed by MRL*lpr/lpr* mice. Eight-week-old MRL*lpr/lpr* female mice were treated with four intraperitoneal injections of an anti-IL-17A antibody (one injection every 4 days), and signs of lymphoproliferation and autoimmunity were evaluated 15 days after the last injection. The control treatments were performed with either PBS or isotype-matched $IgG2_A$. Lymphoproliferation was evaluated in terms of the spleen and lymph node sizes and the expansion of DN T cells in the blood, lymph nodes, and spleen. Autoimmunity was evaluated in terms of serum levels of anti-dsDNA autoantibodies and proteinuria to assess the renal damage. The results showed that treatment with anti-IL-17A antibodies significantly decreased the spleen and lymph node sizes, assessed both as the absolute weight and as a ratio with the whole body weight, compared with the control treatments (Figure 6A). Moreover, the treatment significantly decreased the expansion of DN T cells in the PB, lymph nodes and spleen, as assessed

by flow cytometry (Figure 6B). Finally, the treatment decreased the serum levels of anti-dsDNA autoantibodies, as assessed by ELISA, the proteinuria, and prolonged the overall survival (Figure 7).

Discussion

This work shows that levels of IL-17A, IL-17F, and IL-17AF are increased in ALPS and DALD patients, and the results suggest that these increased levels may contribute to the development of these diseases by increasing the lymphocyte apoptotic defect caused by defective Fas function.

The increased levels were detected in both the serum and in vitro activated PBMCs, and concentrations of IL-17A and IL-17F comparable to those detected in the patients' sera were able to inhibit FICD in Fas-sensitive T cells from healthy donors. Moreover, the patients' sera were able to inhibit FICD, and this effect was reversed by neutralization of IL-17A.

The ALPS and the DALD patients also displayed high levels of IL-1β, which is intriguing since IL-1β plays a key role in induction of IL-17s expression[17]. By contrast, IL-10 and IL-18 were increased in the ALPS but not in the DALD patients, which marks a difference between these diseases. Moreover, both groups of patients displayed decreased levels of $CD4^{+}CD25^{+}FoxP3^{+}$ cells, comprising regulatory T cells, which is in line with the frequent autoimmune manifestations of both diseases (controls median 1.12%, IQR 0.96-1.7; ALPS 0.2%, 0.15-0.4, P= .0003; DALD 0.3%, 0.1-0.38, P=.0007; Supplemental Table1).

The anti-apoptotic effect of IL-17A and IL-17F has not previously been described in human T cells, but it is consistent with a report showing that IL-17A can inhibit AICD, triggered in B cells by crosslinking of the B cells receptor[24].

An intriguing result was that neutralization of IL-17A substantially increased cell apoptosis in the standard FICD assay performed on cells from the patients with high levels of IL-17A, including either ALPS-FAS, or ALPS-UND, or DALD patients, whereas it had no effect on cells from healthy donors. This result indicates that endogenous secretion of IL-17A and, possibly of IL-17F and IL-17AF, may play a role in the apoptotic defect detected by the FIDC assay, at least in this subgroup of patients.

Several studies in mice have compared the sensitivity of Th1, Th2, and Th17 cells to FICD and AICD and have shown that Th17 cells are more resistant to both FICD and AICD than Th1 cells but less than Th2 cells[27]. Our data suggest that the relative resistance to apoptosis of Th17 cells may be ascribed, in part, to autocrine effects of IL-17s. In the ALPS and DALD patients, the defective function of Fas may further increase this resistance to apoptosis and may favor the expansion of this T cell subset and the increased levels of IL-17s, which may in turn favor the development of autoimmunity. This mechanism may also play a role in other autoimmune diseases since high levels of IL-17s similar to those detected in our patients have been described in SLE[24,28], RA[29,30], and psoriasis[31-33]. Moreover, decreased Fas function has been also detected in patients displaying autoimmune diseases different from ALPS and DALD[34-36].

The anti-apototic effect of IL-17s may be partly due to modulation of cFLIP expression since exposure to IL-17A substantially upmodulated $cFLIP_S$ and downmodulated $cFLIP_L$ in Fas-stimulated T cells. This is in line with works showing that $cFLIP_S$ displays higher anti-apoptotic activity than $cFLIP_L$ and that the low sensitivity of Th17 cells to apoptosis may be ascribed to expression of high levels of cFLIPs[37,38].

These data suggest that neutralization of IL-17s may be effective in improving lymphocyte apoptosis in patients with ALPS and DALD. This possibility is also supported by the in vivo experiments in MRL*lpr/lpr* mice, in which IL-17A neutralization had positive effects on the autoimmune features of the diseases and prolonged the animals' life-span. Moreover, it displayed positive effects on the lymphoproliferative features of the diseases which were slight but significant. These therapeutic effects were weaker than those obtained by treating MRL*lpr/lpr* mice with long term chemotherapies, but stronger than those obtained with other immunotherapeutic approaches, such as neutralization of IL-23 or IL-18[39-41]. At this light, it is intriguing that both IL-23 and IL-18 are involved in the IL-17s network, since IL-23 supports Th17 cell expansion and IL-18 potentiates

secretion of IL-17s[39,42-44]. These data may suggest that T cells producing IL-17s may play a pathogenic role in mild forms of the lpr disease.

Thus, IL-17s may play a role similar to that played by OPN, high levels of which in ALPS, DALD, and in MRL*lpr/lpr* mice may favor the development of the disease by reinforcing the lymphocyte apoptotic defect through the direct inhibition of AICD and the induction of secretion of TIMP-1 that can inhibit both FICD and AICD. Moreover, OPN has been shown to induce the secretion of IL-17A and could theoretically play a role in the increased IL-17A secretion observed in our patients[11].

IL-17s appear to play a role in several autoimmune diseases. In animal models, IL-17A is involved in collagen-induced arthritis[45] and in experimental autoimmune encephalomyelitis[21], whereas IL-17F appears to exacerbate the intestinal inflammation observed in dextran sulfate sodium-induced colitis[46]. In humans, a key role has been hypothesized for IL-17A in cell-mediated autoimmune diseases such as MS[22] and in type 1 diabetes mellitus[47]. More recently, however, a role has also been proposed for IL-17s in SLE, in which it could support both the humoral autoimmune response by increasing B cell survival and the inflammation in lupus lesions, with a particular role in renal lesions, by increasing the recruitment of inflammatory cells[24]. In SLE, high IL-17A production has been partly ascribed to DN T cells, which are abundant in the kidney lesions and have been suggested to be terminally differentiated Th17 cells. Similarly, DN T cells infiltrating the kidneys have been shown to produce high levels of IL-17A in MRL*lpr/lpr* mice[48]. In ALPS, the DN T cells have been hypothesized to be exhausted T cells and have been shown to produce high levels of IL-10. IL-10 may play a key role in ALPS, and its high serum levels are included as a minor criterion for ALPS diagnosis[3]. Our experiments did not allow us to detect the production of IL-17s in DN T cells because these cells were lost in our culture conditions. Moreover, DN T cell expansion did not correlate with the serum levels of IL-17s in ALPS patients and were absent in DALD patients, who nevertheless also had high levels of IL-17s. These data suggest that the production of high levels of IL-17s is not strictly dependent on DN T cells in ALPS and DALD patients. Finally, no correlation

was found between serum levels of IL-17s and IL-10 or between the in vitro T cell secretion of IL-17s and IL-10 in our patients.

Recently, the therapeutic use of IL-17A antagonists has been investigated in several autoimmune diseases. LY243982 was the first effective humanized anti-IL-17A mAb to be used in the treatment of RA[49]. More recently, a phase III clinical trial reported the efficacy of a fully human anti-IL-17A antibody (AIN457) in psoriasis, RA, and uveitis[50]. Furthermore, a study evaluating the safety, tolerability, and efficacy of AIN457 in patients with relapsing-remitting MS is in progress (www.clinicaltrials.gov, Novartis).

Patients with ALPS and DALD generally respond to high doses of corticosteroids; however, some of them are refractory and others do not tolerate the side effects of treatment. IL-17A neutralization has been proven to be efficient in other autoimmune diseases and may also offer a targeted and personalized therapeutic option for these patients. Moreover, in ALPS patients, the autoimmune manifestations are often attenuated in adulthood, but they maintain the lymphoproliferative phenotype and are predisposed to developing several types of lymphomas, which is a risk that may be targeted by anti-IL-17 therapy.

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Authorship

Contributions: E.B., N.C., E.O., G.C., E.T., and C.L.G. performed the study; U.R. and A.M. recruited the patients; U.D., and A.C. designed the study and wrote the manuscript; U.D., U.R., and A.C. analyzed the data.

Conflict-of-interest disclosure: The authors declare that they have no competing financial interests.

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Figure Legends

Figure 1. Increased IL-17A, IL-17F and IL-17AF serum levels in ALPS and DALD patients. The black diamonds indicate ALPS (n=18), the black circles indicate DALD (n=18), and the white triangles indicate the healthy controls (HC, n=50). The horizontal bars are the medians, the boxes indicate the interquartile range, the dashed lines indicate the 75th and the thick line indicates the 95th percentile of the HC (Mann-Whitney U-test).

Figure 2. Serum levels of IL-18, IL-10 and IL-1β in ALPS and DALD patients.
Levels of IL-18 (A), IL-10 (B) and IL-1β (C) in ALPS (black diamonds), DALD (black circles), and healthy controls (white triangles). The horizontal bars are the medians, the boxes indicate the interquartile range, the dashed lines in panels A and B indicate the cut off value for ALPS diagnosis. Differences have been tested with the Mann-Whitney U-test.

Figure 3. Increased Th17 cells in ALPS and DALD patients. The proportion of $CD4^+CCR6^+$ cells (A) and $TCR\alpha\beta^+IL\text{-}17A^+$ (B) in PBMCs activated by triggering of CD3+CD28 and cultured with rIL-23. The levels of IL-17A (C) and IL-17F (D) in the culture supernatants are shown. The mean ± SE from 15 patients and 15 HC is shown; * $P < .05$ (Mann-Whitney U-test).

Figure 4. rIL-17s inhibit FICD and increase expression of cFLIP$_S$. FICD was performed in T cells from the healthy controls in the presence or absence of each recombinant cytokine. The results are expressed as % cell survival (A). The mean ± SE from 7 experiments are presented, * $P < .05$ (Wilcoxon's signed rank test). (B) Western Blot analysis of FLIP expression in cells harvested from the FIDC assay (representative of 4 experiments).

Figure 5. Effects of ALPS serum and IL-17A neutralization in FICD. FICD was performed in T cells from healthy donors (A: n=9) or patients (B: ALPS, n=5; diamonds; DALD, n=4; circles) in the presence of either FBS or an ALPS patient's serum. The absolute control cell survival was similar in FBS and in the patient's serum; * $P < .05$ (Wilcoxon's signed rank test).

Figure 6. Effect of IL-17A neutralization in the MRL*lpr/lpr* lymphoproliferative pattern. (A) Representative organs (top) and mean ± SE of organ volumes (bottom) from the differently treated mice (n=8/group). (B) DN T cell expansion is shown in representative cytofluorimetric

plots (top) and the mean ± SE of the data (bottom) from each group are shown * $P < .05$ (Mann-Whitney U-test).

Figure 7. Effect of IL-17A neutralization on the MRL*lpr/lpr* autoimmune phenotype and on lifespan. The mean ± SE of proteinuria (A) and serum anti-dsDNA autoantibodies (B) of the differently treated groups of mice are shown. (C) A Kaplan–Meier survival analysis is shown; anti-IL-17A vs. $IgG2_A$: $P = .0039$; anti-IL-17A vs. vehicle: $P = .0006$.

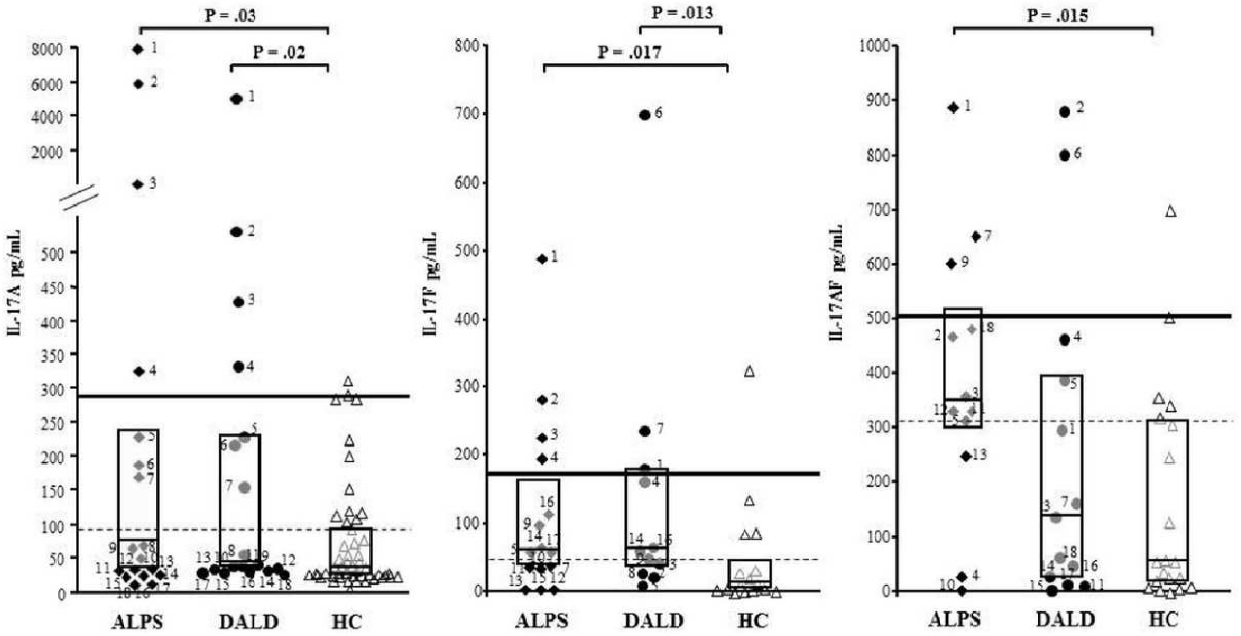
P = .03
P = .02
IL-17A pg/mL
8000
6000
4000
2000
500
450
400
350
300
250
200
150
100
50
0
ALPS
DALD
HC
P = .013
P = .017
IL-17F pg/mL
800
700
600
500
400
300
200
100
0
ALPS
DALD
HC
P = .015
IL-17AF pg/mL
1000
900
800
700
600
500
400
300
200
100
0
ALPS
DALD
HC

Figure 1

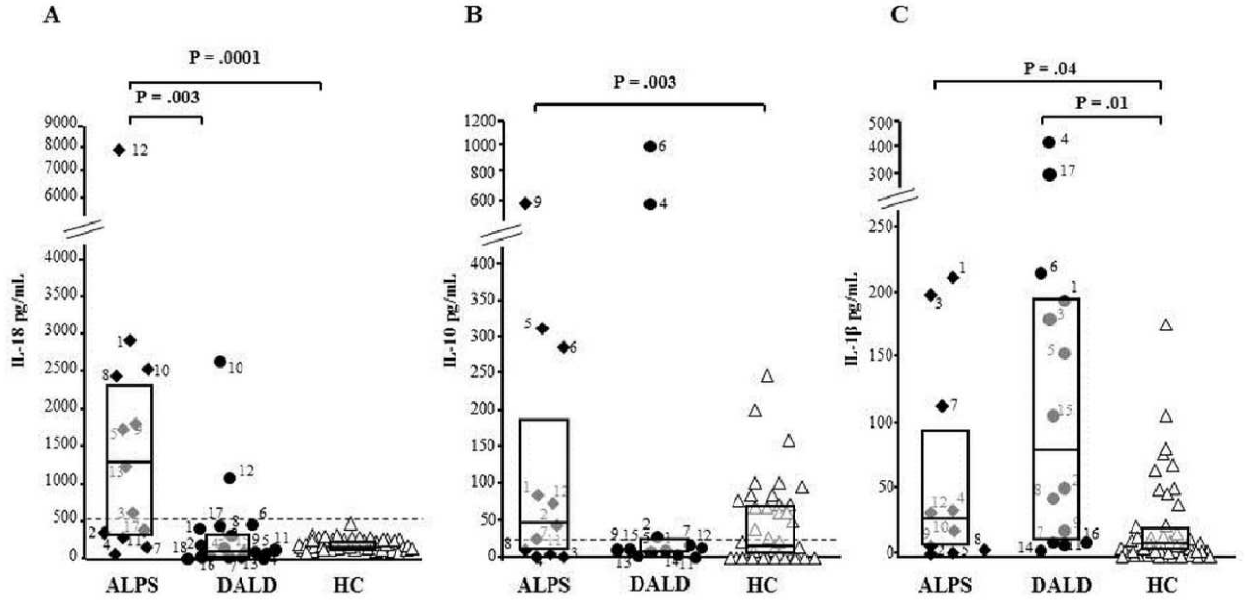
A
P = .0001
P = .003
IL-18 pg/mL
ALPS
DALD
HC
B
P = .003
IL-10 pg/mL
ALPS
DALD
HC
C
P = .04
P = .01
IL-1β pg/mL
ALPS
DALD
HC

Figure 2

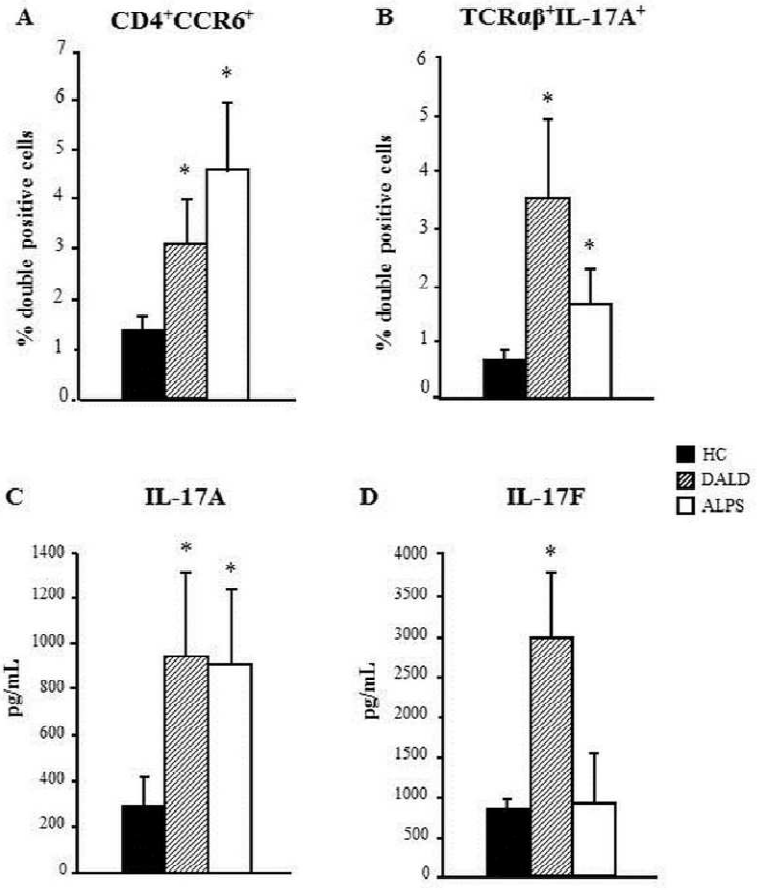
A
CD4+CCR6+
% double positive cells
B
TCRαβ+IL-17A+
% double positive cells
HC
DALD
ALPS
C
IL-17A
pg/mL
D
IL-17F
pg/mL

Figure 3

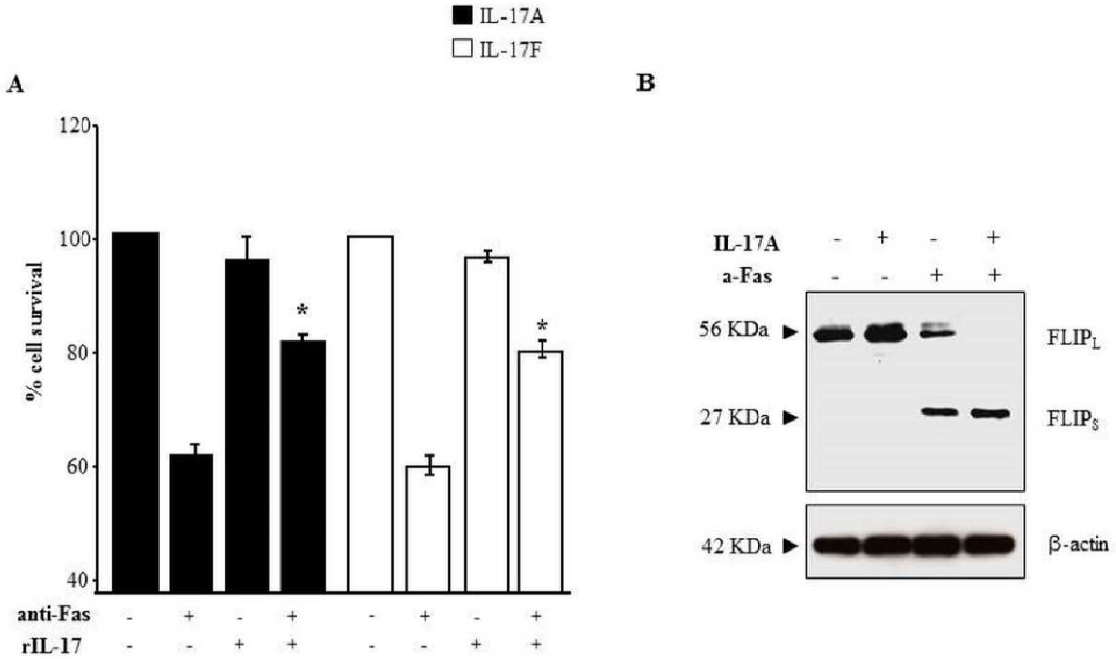
IL-17A
IL-17F
A
B
120
100
80
60
40
% cell survival
*
*
anti-Fas - + - + - + - +
rIL-17 - - + + - - + +
IL-17A - + - +
a-Fas - - + +
56 KDa
27 KDa
42 KDa
FLIPL
FLIPS
β-actin

Figure 4

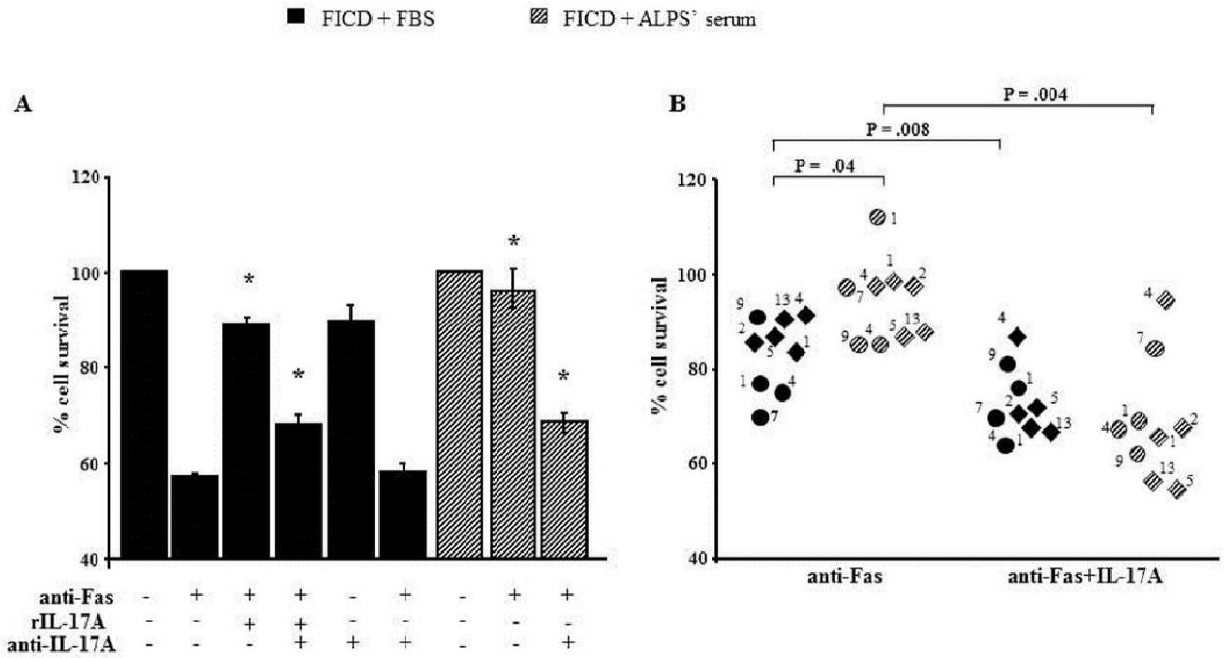
FICD + FBS
FICD + ALPS' serum
A
% cell survival
120
100
80
60
40
anti-Fas - + + + - + - + +
rIL-17A - - + + - - - - -
anti-IL-17A - - - + + + - - +
B
P = .004
P = .008
P = .04
% cell survival
anti-Fas
anti-Fas+IL-17A

Figure 5

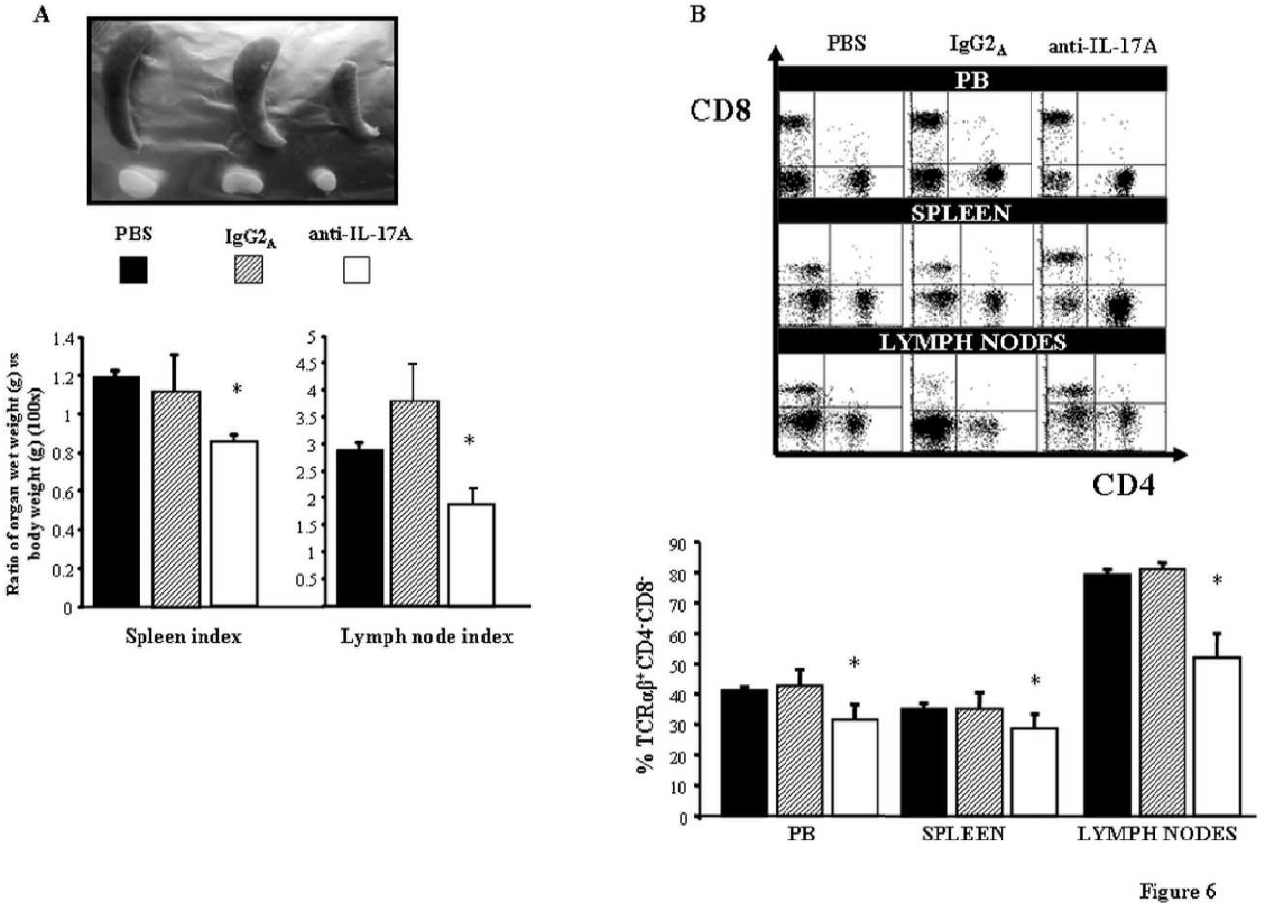
A
PBS
IgG2A
anti-IL-17A
Ratio of organ wet weight (g) vs body weight (g) (100x)
Spleen index
Lymph node index
B
PBS
IgG2A
anti-IL-17A
PB
SPLEEN
LYMPH NODES
CD8
CD4
% TCRαβ+CD4-CD8-
PB
SPLEEN
LYMPH NODES

Figure 6

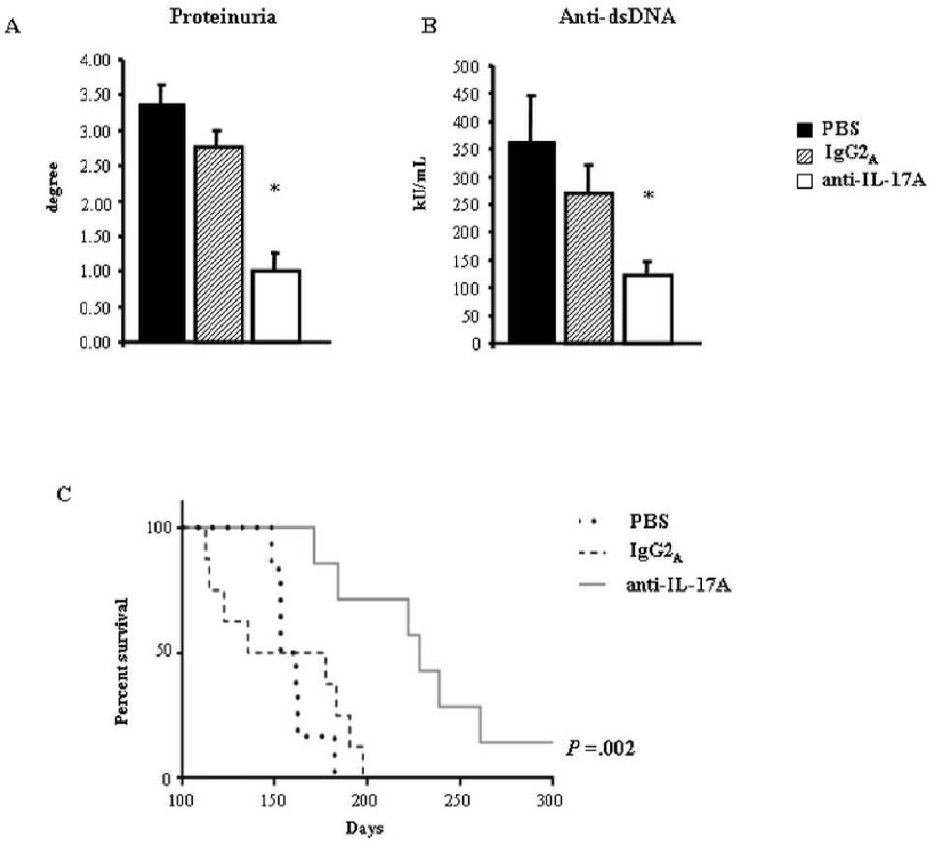
A
Proteinuria
degree
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
B
Anti-dsDNA
kU/mL
500
450
400
350
300
250
200
150
100
50
0
*
PBS
IgG2A
anti-IL-17A
C
Percent survival
100
50
0
100
150
200
250
300
Days
PBS
IgG2A
anti-IL-17A
P =.002

Figure 7

IL-17 protects T cells from apoptosis and contributes to development of ALPS-like phenotypes

Supplemental materials for: Boggio et al

Table S1: Clinical and laboratory features of ALPS and DALD patients (Pt).

Pt no.	ALPS	IL-17A*, pg/mL	IL-17F, pg/mL	IL-17AF, pg/mL	IL-10, pg/mL	IL-18, pg/mL	Gender	Age at diagnosis, y†	DNTs‡, %	Treg§, %	L	S	H	Autoimmunity
1	ALPS FAS	9756	489.9	889.9	87.5	2902.8	F	1+3 m	5	np	-	++	-	ITP, IN
2	ALPS FAS	6031	282.3	469.2	45.5	337.3	M	6	5.6	np	+	++++	++	AHA, ITP, IN
3	ALPS FAS	1171	227.2	358.8	4.9	607.2	F	3	4.3	np	-	+	++	-
4	ALPSU	328.6	196.2	30	4.7	60.9	F	9	10	0.28	-	++	+	ITP
5	ALPS sFAS	231.8	58.9	314	306.3	1655.6	M	2	15	0.42	+++	++++	-	ITP,IN
6	ALPS FAS	189.5	und	und	287.4	na	M	2	7.2	np	++	-	-	-
7	ALPS U	172.3	40.5	655.9	28.4	155	F	2	4.5	0.2	-	++	-	ITP,AHA, eczema
8	ALPS FAS	72.8	und	und	12.9	2356.5	M	3	5.2	np	+++	++	-	ITP
9	ALPS FAS	68.2	98.4	604.5	619	1804.8	M	1+6 m	3.3	np.	++	++++	++	ITP
10	ALPS FAS	53	36.1	6	und	2525.7	M	7	11.5	np	+	-	-	ITP, IN
11	ALPS U	29.7	39	331.8	7.7	271.4	F	7	11	0.15	-	+++	++	ITP
12	ALPS FAS	28.8	6.5	332.7	75.2	8.331	M	5	3.5	np	+	++++	+++	ITP, AHA, IN, arthritis
13	ALPSU	26.5	18.1	251.2	und	1261.8	F	4	20.5	0.1	+	++++	+	IN, ITP, thyroiditis
14	ALPS U	37.1	66.7	und	und	na	M	2	5.4	1.03	+	+++	+++	ITP, IN
15	ALPS U	36.9	3.6	und	und	na	M	11	5.8	0.12	+	+	-	-
16	ALPS U	36.5	115.2	und	und	und	F	6	6.4	0.4	+	-	-	ITP, Eczema
17	ALPS FAS	37.5	58.9	und	und	378.2	F	28	3.7	0.2	+++	++	-	-
18	ALPS U	37.3	und	483.6	na	na	M	13	6.7	p	++	++	-	ITP, IN

Pt no.	DALD	IL-17A, pg/mL	IL-17F, pg/mL	IL-17AF, pg/mL	IL-10, pg/mL	IL-18, pg/mL	Gender	Age at diagnosis,, y	DNTs, %	Treg, %	L	S	H	Autoimmunity
1	DALD	4926	182.3	296.5	12.4	406.6	M	5	0.8	np	+	++	-	IDDM, ITP, IN, alopecia
2	DALD	60.5	29.9	882.9	29.1	190.5	M	5 m.	0.6	0.3	+	++	++++	ITP.
3	DALD	423	43.2	140	na	155	M	2	1.9	np	-	+	-	ITP, IN
4	DALD	333.5	161.4	464.2	619.2	30.8	F	13	1.3	0.9	-	++++	++	-
5	DALD	23.1	12.3	389.1	8.9	73	M	5	1	0.2	++	++	++	AHA
6	DALD	219.1	701.4	802.8	1028.8	449.7	F	8	0.32	np	-	+	-	AHA
7	DALD	157.9	236.1	165	17.8	36.4	F	12	1.1	0.3	+	+	++	AHA, hepatitis
8	DALD	59.9	24.7	und	und	304.5	M	3	1.3	np	+++	++	++	ITP, IN
9	DALD	45	49.6	und	9.7	103.1	M	3	1.5	0.06	+++	+	+	+
10	DALD	43.2	und	und	und	2530. 7	M	11	0.93	0.07	+	-	-	ITP,AHA
11	DALD	41	und	4.9	1.8	122.8	M	2	1.4	np	-	++++	++++	ITP, alopecia
12	DALD	34.4	und	und	6.8	106.7	M	12	1.4	np	++	-	+	ITP,IN, alopecia
13	DALD	33.1	und	und	2.9	36.4	M	9	1.6	1.1	-	-	+	ITP
14	DALD	33.2	61.2	48.5	6.4	58.9	M	11	1.7	0.1	-	++	++++	AHA, ITP, hepatitis
15	DALD	30.3	und	15.8	12.6	87.5	M	22	1.1	np	-	++++	+	arthritis, FSG
16	DALD	37.1	67.4	30.6	und	52.4	M	14	1.5	0.4	+	-	-	-
17	DALD	37.4	und	13.6	und	433.1	M	28	1.4	np	-	++++	-	AHA, ITP
18	DALD	37.5	und	65.4	und	15.5	M	2	1.3	np	+	++	-	-

*: serum levels. †: years. ‡: TCRαβ$^+$CD4$^-$CD8$^-$ T cells. §: CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells
np indicates not performed; na, not available; und, undetectable.
L indicates Lymphoadenopathy; +, single or multiple lymphoadenopathy with diameter of 1-2 cm; ++, single or multiple lymphoadenopathy with diameter of 3-4 cm; +++, single or multiple lymphoadenopathy with diameter greater than 4 cm.
S, Splenomegaly; +, 1-2 cm below costal margin; ++, 3-4 cm below costal margin; +++, 5-6 cm below costal margin; ++++, greater than 6 cm below costal margin.
H, Hepatomegaly; +, 1-2 cm below costal margin; ++, 3-4 cm below costal margin; +++, 5-6 cm below costal margin; ++++, greater than 6 cm below costal margin.
FSG, focal and segmental glomerulosclerosis; AHA, autoimmune hemolitic anemia; ITP, immune trombocytopenic purpura; IN, immune neutropenia; IDDM, Insulin-Dependent Diabetes Mellitus.
No correlations were found between these clinical manifestations and the parameters shown in the table.

6.CONCLUSIONS

The main research projects developed during my PhD work were aimed to understand the pathogenic alterations involved in development of ALPS and DALD, and to identify new possible molecular markers and treatments for these diseases.

Autoimmune Lymphoproliferative Syndrome (ALPS) is a rare genetic disorder of abnormal lymphocyte survival caused by defective Fas mediated apoptosis [100]. Normally, after immune activation against an infectious insult, the immune system is shut-down by several mechanisms. One of them is increase of Fas expression and function on activated B and T lymphocytes, which leads to their apoptotic death upon interaction with Fas-ligand expressed by several cell types, including CTL and NK cells. Patients with ALPS have a defect in this apoptotic pathway, leading to accumulation of polyclonal lymphocytes in the lymph nodes and in the spleen with expansion of TCRαβ DN T cells and frequent development of autoimmune manifestations [101]. This condition is usually caused by mutations in the *FAS* gene (ALPS-FAS), but rare cases due to mutations in other genes including the *FAS-L* (ALPS-FASLG) or *CASP10* gene (ALPS-CASP10) have also been reported. Moreover, the causal gene is not known in a substantial proportion of patients (ALPS-UND). In most cases, the disease is inherited with an autosomal dominant inheritance pattern with incomplete penetrance, but a substantial proportion of patients display an acquired form of the disease due to somatic mutations of *FAS* limited to the DN T cell population (ALPS-sFAS). We also described an incomplete form of ALPS displaying lymphadenomegaly/splenomegaly, autoimmune manifestations and defective Fas function, but lacking the DN T cell expansion. This disease has been named Dianzani Autoimmune Lymphoproliferative Disease (DALD) and has been classified as an ALPS-related disease. DALD displays a genetic component, since most parents of the patients displays the Fas function defect, but it is not caused by mutations of *FAS*, *FAS-L*, or *CASPASE 10*[21,33,35,37].

ALPS patients are heterozygous for the *FAS* mutation, but parents carrying the same mutation are generally healthy. The same observation is true in DALD, where parents typically display defective Fas function, but are otherwise healthy [9,10]. This observation indicates that mutations in genes of the Fas pathway may be necessary but not sufficient for ALPS development and variations in one or more additional genes may influence disease presentation [19].Over the years, our laboratory has been working to identify factors which may work as disease modifiers for ALPS and DALD development in order to detect novel prognostic markers and therapeutic approachesto complement thecorticosteroidtreatmentsused for these diseases.

My laboratory previously suggested a disease-modifying role for the perforin gene involved in FHL, an inherited lymphoproliferative disease displaying some similarities with ALPS, since it correlated certain variants of the perforin gene (*PRF1*) with ALPS and DALD development. It wassuggested that mild heterozygous variations of *PRF1* incapable of inducing FHL may act as susceptibility genes for ALPS and DALD development in subjects with defective Fas function [36,100]. One aim of my PhD work was to extend this observation to the *UNC13D* gene, also involved in FHL, looking for variations in ALPS and DALD patients and assessing its potential role as a disease-modifier gene. The *UNC13D* gene codes for Munc13-4 that is involved in vesicle maturation during exocytosis and secretion of cytolytic granulesin cytotoxic cells with consequent release of perforin. Once granules are tethered to the plasma membrane, a priming step is required to enable fusion of the granule membrane with the plasma membrane. In this priming step, granules interact with a docking complex composed of Munc18-2 and Syntaxin-11. Thus, Munc13-4 triggers the switch of syntaxin-11 from a closed to an open conformation enabling fusion [45]. This analysis detected four rare missense variations in three heterozygous ALPS patients carrying p.Cys112Ser, p.Val781Ile, and a haplotype comprising both p.Ile848Leu and p.Ala995Pro. *In vitro* experiments showed that these variations decreased the Munc13-4 function. Statistical analysis showed that presence of these loss-of-function variations of Munc13-4 may be a risk factor for ALPS, but not DALD, development. Defective function of Fas and Munc13-4 might cooperate in disrupting the ability of the immune system to shut off and interfere with the anti-viral response. These processes involve both Fas and cytotoxic cell function, which are crucial for the clearance of virus-infected cells and the fratricide of activated immune cells [101]. Persistence of viral infection and an inability to switch off the immune response may contribute to the lymphocyte accumulation and the autoimmune reactions displayed by ALPS patients.

Since the work on perforin and Munc13-4 indicated that defective function of cytotoxic cells may favor ALPS development, the work was extended to SAP involved in activation of cytotoxic cells and development of XLP, an inherited lymphoproliferative disease with some similarities with ALPS. XLP is characterized byinability to mount an effective immune response to Epstein–Barrvirus (EBV) with fulminant infectious mononucleosis, polyclonallymphoproliferation, and dysgammaglobulinaemiathat can progress to hypogammaglobulinaemia. A rarer manifestation is the development of autoimmune disorders such as vasculitis, colitis, and psoriasis. The phenotype of SAP-deficient mice recapitulates several features of XLP [102]. SAP is an intracellular adaptor protein including a single Src homology 2 (SH2) domain and is expressed by T cells, NK cells, NKT cells, eosinophils, platelets, and some B-cell populations [103]. SAP regulates the function of many receptors belonging to the SLAM family by binding to their cytoplasmic tails and serving as a

docking platform for signaling molecules. Receptors of the SLAM family have been implicated in the regulation of NK cell cytotoxicity, NKT cell development, Th2 cell priming, isotype switching, and maintenance of long-term antibody-secreting cells. Komori et al. [104] observed that, in a breeding colony of MRL*lpr/lpr* mice, a spontaneous A insertion at the 21st codon of *SH2D1A* (first exon) causing a frame-shift resulted in defective expression of SAP, and protection from development of the *lpr* disease with striking reduction of hypergammaglobulinemia, autoantibody production, DN T-cells counts, lymphadenopathy, splenomegaly, and pathological indexes of glomerulonephritis and vasculitis. Furthermore, a general role of SAP in autoimmunity has been suggested by the finding that SAP-deficient mice are protected also from EAE and pristane-induced lupus [105]. These data support the possibility of an opposite epistatic relationship between Fas and SAP expression.

To assess this possibility, we sequenced the coding and regolatory regions of the *SAP* gene in ALPS or DALD patients. We found that ALPS and DALD patients displayed an increased frequency of the -346T allele that lacked the -346C methylation site and correlated with increased expression of SAP in NK and CD8$^+$ T cells and decreased secretion of IFN-γ. These data suggest that high SAP expression favors development of ALPS and DALD in humans, and confirm the data reported on MRL*lpr/lpr* mice. This work opens the way to the possibility that partial pharmacological inhibition of SAP might be beneficial to control these diseases.Interestingly, we found that association with -346T was detected only in males, which might reflect differential use of the -346C methylation site in males and females. Intriguingly, Maric et al. [106] showed that lymph nodes from male ALPS patients frequently displayed histopathological features of sinus histiocytosis with massive lymphadenopathy, that were rare in female ALPS patients, underlining a gender influence in the ALPS picture.

Previous work of our laboratory showed that other complementary factors which might be involved in development of ALPS and DALD are osteopontin [34] and TIMP-1[107], since their levels are elevated in these patients. Since high levels of these molecules inhibit lymphocyte apoptosis, they may contribute to ALPS and DALD development in subjects with a hypofunctional Fas system by worsening the genetic apoptosis defect [34,107]. These data prompted our interest on the role of IL-17 in ALPS and DALD development, since the literature showed that osteopontin is involved in development of Th17 cells[108], characterized by secretion of IL-17A and IL-17F, which can be secreted as homodimers or IL-17AF heterodimers[12]. IL-17A and IL17F share several biological activities including neutrophil recruitment, induction of proinflammatory cytokines, chemokines, and metalloproteinases, and involvement in autoimmunity[109]. However, IL-17A displays higher proinflammatory activity than IL-17F and they are differently expressed in several autoimmune and

allergic diseases[110]. Moreover, their receptor usage is partly different and their secretion is independently regulated in Th cells[111,112]. In SLE, high IL-17A production has been partly ascribed to DN T cells, which are abundant in the kidney lesions and have been suggested to be terminally differentiated Th17 cells. Similarly, DN T cells infiltrating the kidneys have been shown to produce high levels of IL-17A in MRL*lpr/lpr* mice[113].We thus decided to assess whether IL-17 was involved in the pathogenic mechanisms of ALPS and DALD.

We first showed that levels of IL-17A, IL-17F, and IL-17AF are increased in ALPS and DALD patients. These high levels were detected in both the serum and in vitro activated PBMC and did not correlate with the expansion of DN T cell or the levels of other cytokines, such as IL-10 and IL-18,known to be increased in ALPS.The concentrations of IL-17A and IL-17F detected in the patients' sera were able to inhibit Fas-induced apoptosis in Fas-sensitive T cells from healthy donors and deregulated the expression of FLIPs which are key regulators of Fas-induced apoptosis. Moreover, the patients' sera were able to inhibit this Fas-induced cell death and IL-17A neutralization restored the death of lymphocytes. These data suggest that high IL17Aand IL-17F levels in ALPS and DALD patients may worsen the Fas defective function and play a role in the development of these diseases. These data suggest that neutralization of these IL-17s may be effective in improving lymphocyte apoptosis in patients with ALPS and DALD, which has been formally proven in MRL*lpr/lpr* mice, where treatment with anti- IL-17A antibodies had a substantial effect in ameliorating the autoimmune manifestations and prolonging the animals' life-span, and a mild effect on the lymphoproliferative features, evaluated in terms of lymph nodes/spleen size and DN T cell expansion. IL-17A neutralization has been proven to be efficient in other autoimmune diseases and may also offer a targeted and personalized therapeutic option for these patients.

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