Deregulation of Fas ligand expression as a novel cause of autoimmune lymphoproliferative syndrome-like disease

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ABSTRACT

Autoimmune lymphoproliferative syndrome is frequently caused by mutations in genes involved in the Fas death receptor pathway, but for 20-30% of patients the genetic defect is unknown. We observed that treatment of healthy T cells with interleukin-12 induces upregulation of Fas ligand and Fas ligand-dependent apoptosis. Consistently, interleukin-12 could not induce apoptosis in Fas ligand-deficient T cells from patients with autoimmune lymphoproliferative syndrome. We hypothesized that defects in the interleukin-12 signaling pathway may cause a similar phenotype as that caused by mutations of the Fas ligand gene. To test this, we analyzed 20 patients with autoimmune lymphoproliferative syndrome of unknown cause by whole-exome sequencing. We identified a homozygous nonsense mutation (c.698G>A, p.R212*) in the interleukin-12/interleukin-23 receptor-component *IL12RB1* in one of these patients. The mutation led to IL12RB1 protein truncation and loss of cell surface expression. Interleukin-12 and -23 signaling was completely abrogated as demonstrated by deficient STAT4 phosphorylation and interferon γ production. Interleukin-12-mediated expression of membrane-bound and soluble Fas ligand was lacking and basal expression was much lower than in healthy controls. The patient presented with the classical symptoms of autoimmune lymphoproliferative syndrome: chronic non-malignant, non-infectious lymphadenopathy, splenomegaly, hepatomegaly, elevated numbers of double-negative T cells, autoimmune cytopenias, and increased levels of vitamin B12 and interleukin-10. Sanger sequencing and whole-exome sequencing excluded the presence of germline or somatic mutations in genes known to be associated with the autoimmune lymphoproliferative syndrome. Our data suggest that deficient regulation of Fas ligand expression by regulators such as the interleukin-12 signaling pathway may be an alternative cause of autoimmune lymphoproliferative syndrome-like disease.

Introduction

The autoimmune lymphoproliferative syndrome (ALPS, Canale-Smith syndrome) of early childhood[1,2] is caused by disturbance of apoptotic signaling via the Fas death receptor pathway which primarily compromises lymphocyte homeostasis.[3,4] Fas is a member of the tumor necrosis factor receptor superfamily. At the cell surface Fas is activated upon binding of its specific ligand (Fas ligand, FasL). This triggers the intracellular activation of caspase-8 and -10 to start a proteolytic cascade resulting in cell death.[5] Due to deficient apoptosis, T lymphocytes are inefficiently cleared resulting in chronic lymphadenopathy, hepatosplenomegaly, autoimmune cytopenias, and elevated numbers of terminally differentiated, activated, otherwise rare double-negative T cells (DNT cells: $CD3^+$, $TCR\alpha/\beta^+$,$CD4^-CD8^-$). Malignancies, especially B-cell lymphomas, may develop later in life.[6]

ALPS is a genetically heterogeneous disease. Most patients harbor heterozygous, autosomal dominant or recessive *FAS* germline mutations (ALPS-FAS[7]). Ninety-seven unique mutations are registered in the database of ALPS mutations at the National Institute of Allergy and Infectious Disease, *www.niaid.nih.gov*). However, somatic FAS mutations have also been reported (ALPS-sFAS).[6,8-11] Homozygous or compound heterozygous FAS mutations result in a clinically more severe phenotype. Less frequently, *FASLG* mutations (ALPS-FASLG)[12-16] or *CASP10* mutations (ALPS-CASP10)[17] have been detected. Similar symptoms are caused by mutations in *CASP8* (caspase-8 deficiency state), *KRAS* or *NRAS* (RAS-associated ALPS-like disease). These cases are not classified as ALPS, because caspase-8 deficiency state is additionally characterized by disturbed T- cell activation and immunodeficiency and RAS-associated ALPS-like disease by monocytosis.[18-21] For 20-30% of patients with the clinical picture of ALPS, however, the genetic cause is still unknown (termed ALPS-U cases).[2]

It is plausible that Fas pathway regulating factors may present novel candidates causing ALPS and potential drug targets.

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For instance, forced expression of the Fas regulating miR-146a caused an immune disorder similar to ALPS in transgenic mice.[22] One factor known to regulate expression of both Fas and FasL is interleukin-12 (IL12).[23-30] This cytokine mediates its effects via binding to a heterodimeric receptor composed of the IL12 receptors β1 (IL12RB1) and β2 (IL12RB2) and subsequent activation of JAK/STAT signaling.[31] The IL12 receptor is mainly expressed on T lymphocytes and natural killer (NK) cells. IL12RB1 deficiency is associated with Mendelian susceptibility to mycobacterial diseases.[32-35] These diseases predispose affected individuals to mycobacterial infections (tuberculosis) due to disturbance of IL12/IL23/IFNγ signaling. IL12RB1 also associates with IL23R to form the IL23 receptor. Phagocytic cells release both IL12 and IL23 in response to non-viral infections to induce the secretion of interferon (IFN)γ by T and NK cells. This in turn stimulates phagocytes to eliminate intracellular pathogens and activation of IFNγ–secreting T helper cells. The IL12 signaling pathway is known to regulate FasL expression on activated T cells and knockout of *IL12RB2* predisposes mice to spontaneous autoimmunity, lymphoproliferation and B-cell malignancies.[36] In the present study we identified a patient with a classical ALPS phenotype that was associated with a truncating nonsense mutation in IL12RB1 and loss of IL12/IL23-mediated signaling.

Methods

Study cohorts and DNA isolation

Twenty-six ALPS patients, relatives and healthy controls were enrolled in the study. Written informed consent was obtained from all participants. Experiments were approved by the Ethical Review Boards of Hadassah, the Israeli Ministry of Health and the local Ethics committee of the University of Düsseldorf. Mononuclear cells were derived from peripheral blood by Ficoll (Biochrom, Berlin, Germany) density centrifugation. DNT cells were magnetically selected employing the double-negative T-cell isolation kit (Miltenyi, Bergisch-Gladbach, Germany). Genomic DNA was isolated from whole blood or DNT cells using the DNA blood kit (Qiagen, Hilden, Germany).

Whole-exome sequencing and data analysis

After exclusion of mutations in known ALPS-associated genes by targeted Sanger sequencing (*Online Supplementary Methods, Online Supplementary Table S1*) whole-exome sequencing was carried out as described elsewhere.[37] In brief, sequencing libraries of 350 bp fragments were generated from sheared genomic DNA. Exome capture was performed using the SeqCap EZ Exome Library 2.0 kit (Roche/Nimblegen, Madison, WI, USA). One hundred base-pair, single-read sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA, USA).

Sequencing data were aligned against the human reference genome hg19 (GRCh37, statistics provided in *Online Supplementary Table S2*) and converted using Samtools.[38] Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA, USA).[39] Single nucleotide variations, small insertions and deletions were annotated using Variant Effect Predictor[40] (based on Ensemble database v70). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy). STRING 9.1[41] was used to identify high confidence (≥0.900) Fas pathway interaction partners (*Online Supplementary Table S3*).

Primary T-cell culture

Primary T cells were cultured in RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% fetal calf serum, 100 μg gentamycin (Life Technologies) and 30 U/mL IL2 (Miltenyi). They were activated with 7 μg/mL phytohemagglutinin (Life Technologies) for 4 days.

Immunophenotyping and enzyme-linked immunosorbent assays

DNT cells in peripheral blood were measured using a FACSCalibur equipped with CellQuestPro software (Becton Dickinson, BD, Heidelberg, Germany) employing anti-CD3, anti-TCRαβ (both from BD), anti-CD4 and anti-CD8 (both from Miltenyi) antibodies. Immunophenotyping was performed using: anti-B220, anti-HLA-DR, anti-CD27, anti-CD19, anti-CD25 (all from BD) and anti-CD45R (Beckman Coulter, Krefeld, Germany). Expression of Fas, FasL and IL12RB1 was measured using anti-CD95 (BD), anti-CD178/FasL (Miltenyi) and anti-CD212 antibodies (BD). FasL, IL10 and IFNγ levels in plasma and cell culture supernatants of activated T cells were measured by enzyme-linked immunosorbent assays (R&D-Systems, Wiesbaden, Germany) employing an Infinite M200 microplate reader equipped with Magellan software (Tecan, Maennedorf, Switzerland). T cells were stimulated with IL12, IL23 and IL2/IL27 (Miltenyi) as indicated.

Measurement of apoptosis

Activated primary T cells were stimulated with recombinant SuperFasL (100 ng/mL, Enzo Life Sciences, Loerrach, Germany), 1 μM staurosporine (LC Laboratories, Woburn, MA, USA), IL12 (50-200 ng/mL, Miltenyi) or left untreated. After the indicated time apoptosis was measured by annexinV-FITC (BD) and propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) staining and flow cytometry. (Further methods in the *Online Supplementary Material*).

Results

Interleukin-12 induces upregulation of FasL and FasL-dependent apoptosis in healthy T cells, whereas FasL-deficient T cells from patients with autoimmune lymphoproliferative syndrome lack this response

Of 26 analyzed ALPS cases, 20 had no known ALPS-associated mutation. Four patients harbored heterozygous germline mutations in the Fas receptor gene. Two siblings had a homozygous truncating *FASLG* mutation (g.172628545insT, p.P69Afs*75) that led to loss of FasL surface expression (Figure 1A).[16] Heterozygous carriers of this mutation showed a similar expression of FasL as healthy controls. In response to prolonged IL12 treatment healthy T cells upregulated FasL (Figure 1B) and died apoptotically (Figure 1C). In spite of normal expression of IL12 receptor components (*Online Supplementary Figure S1* and *data not shown*) this response was completely lacking in the two individuals harboring the g.172628545insT, p.P69Afs*75 mutation indicating that IL12 induces apoptosis via FasL. Downstream of the Fas receptor these patients and the heterozygous carrier had a functional apoptotic pathway as could be demonstrated by the response of their primary T cells *in vitro* to application of recombinant FasL to the cell culture medium (Figure 1D). These results suggest that IL12 induces FasL-dependent apoptosis and that T cells deficient in FasL expression or

IL12 signaling may be protected against this physiological apoptosis trigger.

Identification of a homozygous c.698G>A, p.R212 mutation in the* IL12RB1 *gene*

To analyze whether mutations in the IL12 pathways or related genes may cause a phenotype similar to ALPS, we sequenced the exomes of the remaining 20 ALPS-U patients who had classical ALPS symptoms without a known genetic cause, and their relatives. Sanger sequencing of all exons and exon/intron boundaries of *FAS*, *FASLG* and *CASP10* was used to exclude classical disease-causing germline or somatic mutations.

By whole-exome sequencing we identified an *IL12RB1* mutation in one of the ALPS families. A KEGG-based protein interaction analysis interface of our in-house developed proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy) gave this candidate disease-causing mutation highest priority because of its predicted interaction with FasL (Figure 2A, *Online Supplementary Figure S2*). None of the other six candidate genes derived by our sequencing data filter strategy interacted with FasL, Fas, Caspase-10 or other immediate components of the signaling cascade. STRING 9.1[41] was used to identify high confidence (≥0.900) interaction partners of the Fas pathway.

The mutation on chromosome 19:18186625 corresponded to c.698G>A, p.R212* of *IL12RB1* and led to a stop codon gain and premature truncation of the receptor component in its extracellular domain (*Online Supplementary Tables S4* and *S5*, Figure 2A). Sanger sequencing of *IL12RB1* revealed that parents and siblings were heterozygous mutation carriers, whereas the patient was homozygous (Figure 2B,C). This mutation was not observed previously in the 1000 Genomes, HapMap, Exome Variant Server (NHLBI GO Exome Sequencing Project, Seattle WA; http://evs.gs.washington.edu/EVS/) data sets or in our in-house database of more than 300 whole-exome data sets.

As the mutated sequence seemed to encode a truncated protein that lacks the transmembrane domain necessary for membrane anchorage, we carried out FACS analyses of the patient's lymphocytes to test for IL12RB1 expression on the cell surface. To this end, primary lymphocytes from the patient and healthy individuals were stimulated for 4 days with phytohemagglutinin/IL2. Whereas lympho-

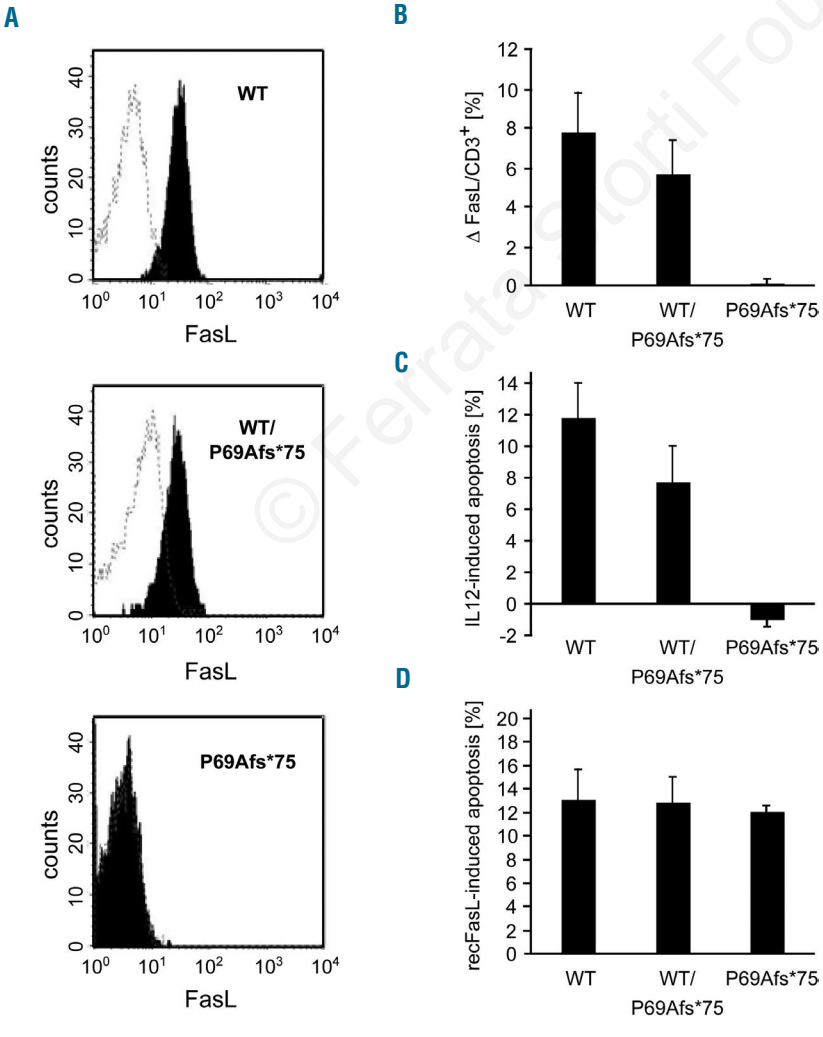

Figure 1. (A) Premature protein truncation due to a homozygous insertion of one base pair in exon 1 of *FASLG* (g.172628545insT, p.P69Afs*75) leads to loss of FasL expression on the surface of $CD3^+$ cells (lower panel). A heterozygous carrier (middle panel) expresses similar FasL levels as the wild-type control (upper panel). Representative flow cytometric measurements of FasL expression on primary T cells after activation with phytohemagglutinin (7 μg/mL) in the presence of IL2 (30 U/mL) for 4 days are shown. (B) Individuals carrying the homozygous *FASLG* mutation (g.172628545insT, p.P69Afs*75) are deficient in upregulation of FasL in response to IL12. Primary T cells of a healthy wild-type control, a heterozygous carrier and a patient with the homozygous *FASLG* (g.172628545insT, p.P69Afs*75) mutation were activated as decribed in (A) and treated with 100 ng/mL IL12 or left untreated for 2 further days. FasL expression on $CD3^+$ cells was measured employing flow cytometry. The percentage of FasL-expressing $CD3^+$ cells specifically induced in IL12-treated compared to untreated cells is shown. (C) Cultivated patient's T cells are resistant to apoptosis induced by stimulation with IL12. T cells were activated as in (A). Apoptosis was induced by incubation with 100 ng/mL IL12 for 2 days and measured employing flow cytometric detection of annexin V-FITC and propidium iodide. The difference in the apoptosis rate compared to that of an untreated control is depicted. Specific apoptosis ranged from 4-12% in the healthy controls between comparable experiments and was absent in the patient's cells. (D) T cells were activated by phytohemagglutinin /IL2 treatment as described in (A). Fas receptor-mediated apoptosis was triggered by application of 100 ng/mL optimized and pre-oligomerized recombinant FasL for 16 h and apoptosis was measured as in (C). In (B-D) mean values and standard deviations of representative experiments repeated at least three times and carried out in duplicate are shown. Similar results were obtained using samples from two individuals with homozygous *FASLG* (g.172628545insT, p.P69Afs*75) mutation and five wild-type controls.

cytes from healthy individuals upregulated IL12RB1 surface expression upon activation, IL12RB1 remained absent in the patient's lymphocytes (Figure 3A). Similar results were gained employing activated T lymphocytes from the patient and healthy controls immortalized by Herpes virus saimirii (*Online Supplementary Figure S3*). Expression of the IL12 receptor component IL12RB2 and the IL23 receptor component IL23R was unaffected by the mutation of their heterodimerization partner IL12RB1 and was upregulated by phytohemagglutinin activation in both wild-type and IL12RB1 mutant cells (*Online Supplementary Figure S4*). To confirm the lack of full-length IL12RB1 expression we performed immunoblot analysis employing an antibody directed against the C-terminal region of IL12RB1. Immortalized T lymphocytes from the patient and healthy controls were lysed and western blot analysis carried out. Expression was further compared to that of freshly isolated donor lymphocytes as well as lymphocyte cell lines. Full-length IL12RB1 expression was absent from the cells derived from the patient (Figure 3B and *data not shown*).

The homozygous c.698G>A, p.R212 mutation in the* IL12RB1 *gene was associated with an autoimmune lymphoploliferative syndrome-like phenotype*

The male patient harboring the homozygous *IL12RB1* c.698G>A, p.R212* mutation originated from a consanguineous family of Palestinian descent. He was referred in 1996 at the age of 4 years because of the suspicion of lymphoma. In the follow up of this patient for more than 16 years he presented with classical clinical features for the diagnosis of ALPS (Table 1, Figure 4A). The two criteria required to diagnose ALPS – (i) chronic non-malignant, non-infectious lymphadenopathy and splenomegaly and (ii) elevation of the number of DNT cells – were both fulfilled. DNT cells were persistently increased (Table 1, Figure 4B). In addition, the patient exhibited typical secondary characteristics such as hepatomegaly, autoimmune cytopenias (hemolytic anemia) with polyclonal hypergammaglobulinemia, and persistently elevated levels of vitamin B12 and IL10. He had one short episode of Salmonella bacteria group B infection at the age of 11 that did not recur. No indication of active infections by mycoplasma or viruses (Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus, hepatitis B and C viruses) was apparent.

The heterozygous parents and siblings appeared clinically normal, although immune phenotyping revealed increased DNT cell counts in two of them (Table 2).

In vitro the apoptotic response of the patient's lymphocytes to treatment with recombinant FasL and a classical apoptosis-inducing agent (staurosporine) was similar to that of age- and gender-matched healthy blood donors [FasL-induced apoptosis: 58% ± 5% (patient), 57% ± 4% (healthy control); staurosporine-induced apoptosis: 86% ± 6% (patient), 86% ± 7% (healthy control)]. This demon-

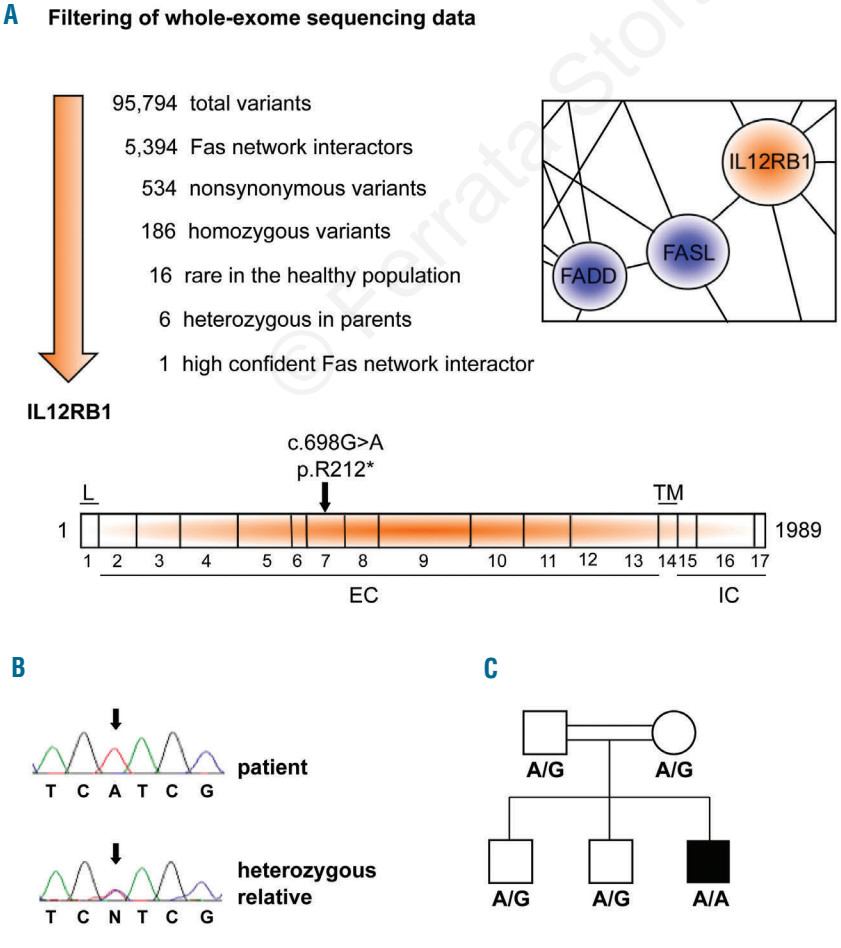

Figure 2. Identification of a homozygous stop mutation in the *IL12RB1* gene in a patient of the analyzed ALPS cohort. (A) *Left panel*: Overview of the strategy employed to filter whole-exome sequencing data resulting in *IL12RB1* as the only candidate gene. *Right panel*: Detail of the network of interactions between members of the Fas pathway and IL12RB1. STRING 9.1 was used as a resource for protein-protein interactions. High confidence links (≥0.900) between proteins are shown. Members of the Fas signaling pathway are shown in blue. IL12RB1 as a gene product bearing a homozygous mutation in the patient but not in unaffected family members is shown in red. IL12RB1 was the only candidate showing a direct high confidence interaction with FasL. *Lower panel*: Schematic drawing of the IL12RB1 protein comprising 1989 nucleotides coding for 662 amino acids. The patient's mutation led to a premature stop at codon 212 (exon 7, c.698G>A, p.R212*; L: leader peptide; EC: extracellular domain; TM: transmembrane domain; IC: intracellular domain). Exons are separated by vertical bars and numbered from 1 to 17. (B) Sanger sequencing of *IL12RB1* confirmed a heterozygous mutation (c.698G>A) in the parents and the siblings and a homozygous mutation in the patient. Representative chromatograms of a heterozygous relative and the patient are shown. (C) Family pedigree of the affected patient. The parents and two siblings were asymptomatic, heterozygous carriers of the *IL12RB1* c.698G>A mutation (genotype A/G). The patient represents the only affected, homozygous carrier of this mutation (genotype A/A). The parents are consanguineous.

strated functional apoptotic signaling downstream of the Fas receptor (Figure 4C) and indicated a FasL or related defect.

To analyze whether the defect in *IL12RB1* affects FasL signaling, we first tested protein expression of FasL. Activation and expansion of T cells usually leads to upregulation of Fas signaling pathway components. However, in the absence of *IL12RB1* expression the patient's T lymphocytes showed a significantly lower expression of both membrane-bound and soluble FasL protein compared to that of healthy controls (Figure 4D,E and *Online Supplementary Figure S5*).

The IL12RB1 c.698G>A, p.R212* mutation abrogates responsiveness of T cells to interleukin-12

To test whether lack of IL12RB1 expression affects IL12 signaling we analyzed phosphorylation of STAT4, a crucial downstream component of the pathway (Figure 5). To this end, we incubated activated T lymphocytes from the patient and a healthy control with or without IL12 for up to 72 h. Immunoblot analyses of total and phosphorylated STAT4 protein demonstrated a relative increase of phosphorylated STAT4 in the healthy control in response to IL12. In contrast, this response was completely absent in the patient's lymphocytes indicating deficient IL12 signaling.

STAT4 activation eventually leads to transcription and production of IFNγ. To test whether this is deficient in the patient we measured the induction of *IFNG* transcription by quantitative real-time polymerase chain reaction after 24 and 48 h of treatment with IL12 (Figure 6A). Whereas the wild-type control rapidly and strongly upregulated *IFNG* by about 500 fold, there was no response in the IL12RB1 c.698G>A, p.R212* mutated cells. This could be confirmed on the protein level employing enzyme-linked immunosorbent assays (Figure 6B). The cells were stimulated with IL12, IL23 or IL2/IL27. In contrast to wild-type T cells, there was no response of the IL12RB1 c.698G>A, p.R212* mutated cells to treatment with either IL12 or IL23. An upregulation of IFNγ was only detectable after treatment with IL27, which uses a different receptor. Similarly, *FASLG* mRNA levels analyzed by real-time polymerase chain reaction (Figure 6C) and FasL protein expression analyzed by FACS (Figure 6D) were not induced in response to IL12 treatment in the IL12RB1 c.698G>A, p.R212* mutated cells, in contrast to the wild-type control.

Finally, we tested the responsiveness of the patient's primary lymphocytes to apoptosis mediated by prolonged IL12 treatment (Figure 6E). Activated T cells were treated with IL12 for 3 days. Specific apoptosis, measured by annexin V-FITC staining, was induced upon incubation with IL12 in the healthy controls, but not in the patient's cells.

Discussion

Although ALPS is frequently caused by mutations in known genes, such as *FAS*, *FASLG* or *CASP10*, in 20-30% of cases the defect is still unknown. It is highly likely that defects in or overexpression of regulators of these genes such as miR-146a[22] could result in an ALPS-like phenotype and account for a not yet defined percentage of ALPS-U cases. In the present study we identified an *IL12RB1* mutation and the IL12 signaling pathway as such an alternative cause of an ALPS-like phenotype through regulation of FasL expression. Previously it was shown that activation of T and NK cells by IL12 results in upregulation of FasL.[23-26,28,42] For instance, Yu *et al.* showed that dendritic cell-derived IL12 is involved in upregulation of FasL on NK cells leading to cell death.[25] Moreover, in the absence of antigen, IL12 induces apoptosis of T cells *via* upregulation of FasL which can be blocked by anti-FasL antibodies.[26] In line with this, we found that primary human T cells deficient in FasL expression were resistant to apoptosis induced by IL12. *In vivo* models demonstrated that IL12 induces apoptosis of $CD8^+$ tumor-resident T cells *via* FasL and showed that IFNγ was necessary for this effect.[42] IL12 also regulates Fas expression. Zhou *et al.* showed that IL12 specifically induces Fas promoter activity in breast carcinoma, osteosarcoma and Ewing tumor cells.[29] IL12 treat-

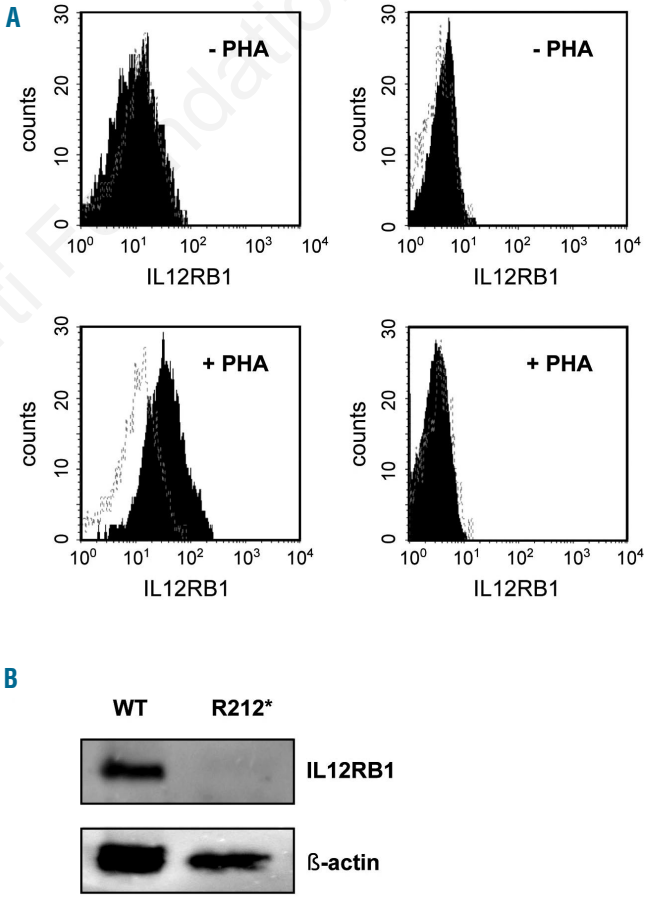

Figure 3. The homozygous *Il12RB1* mutation c.698G>A, p.R212* leads to absence of the receptor on the surface of the patient's primary T cells. (A) Flow cytometric measurement of activation induced IL12RB1 surface expression on primary T lymphocytes of the patient (*right panels*) compared to a healthy control (*left panels*) after 4 days of treatment with phytohemagglutinin (PHA) and IL2 as described in Figure 1A (*lower panels*) or without activation by PHA (*upper panels*). (B) Immunoblot analysis of IL12RB1 protein expression. Protein extracts were prepared from PHA/IL2 activated, HVS-immortalized T lymphocytes from the patient and a healthy control. Western blot analysis was carried out employing an IL12RB1-specific antibody using β-actin as a loading control. (A, B) Results from representative experiments repeated three times are shown. Similar results were obtained using samples from three other wild-type controls.

Table 1. Clinical and laboratory data of the IL12RB1-deficient patient.

Trait	Patient's values	Normal values	Unit/value
Age at presentation, years	4		years
Gender	Male		
Clinical findings on presentation	Lymphadenopathy, splenomegaly, hepatomegaly, night sweats, pruritus	none	na
Clinical findings during the course	Progressive lymphadenopathy and splenomegaly, arthralgia, skin rash (leukocytoclastic vasculitis), livido reticularis	none	na
Laboratory findings during the course	Anemia, intermittent leukocytosis, polyclonal hypergammaglobulinemia	none	na
Detected infections	One episode of *Salmonella ssp.* (lymph nodes) and another episode of *Helicobacter pylori* infection	none	na
Autoantibodies	ASCA, anti-smooth muscle (+1), Coombs (1+), ANA (1:100), rheumatoid factor (182), microcytic anemia (MCV 63)	negative	na
Hemoglobin	9	12-16	g/dL
Double-negative T cells	Chronically elevated, 8-27	<2.5 (range, PB)	%,
$B220^+$	92	<60	% (of DNT)
$CD3^+CD25^+$/HLA-DR^+	0.58	>1	(ratio)
$CD27^+$	12%	>15%	% (of B cells)
Vitamin B12	618-1002	200-800	pg/mL (range)
Platelets	510	150-450	$x10^9$/L
Neutrophils	9.7	2.5-7.5	$x10^9$/L
Plasma IL10	45	<20	pg/mL
Soluble FasL	<60	≤200	pg/mL

na: not applicable; ASCA: anti-Saccharomyces cerevisiae *antibody; ANA: anti-nuclear antibodies; PB: peripheral blood.*

ment stimulated nuclear factor κB, and the κB-Sp1 motif (-195 to -286) in the Fas promoter sequence was essential for the activation of Fas. Lafleur *et al.* demonstrated that IL12 and IL12 gene transfer upregulates Fas expression on osteosarcoma and breast cancer cells.[30]

Consistently, we identified a homozygous stop mutation that led to loss of cell surface expression of the IL12/IL23 receptor component IL12RB1 in a patient presenting with classical ALPS symptoms in the absence of germline or somatic mutations in known ALPS genes. The patient presented with chronic non-malignant, non-infectious lymphadenopathy, splenomegaly, hepatomegaly, persistent elevation of DNT cell counts, autoimmune cytopenias with polyclonal hypergammaglobulinemia, and persistently increased levels of vitamin B12 and IL10.

It might be reasoned that the observed autoimmunity and lymphoproliferation are likely a side-effect of recurrent infections, because *IL12RB1* mutations have previously been associated with a predisposition to mycobacterial infections. However, the patient experienced only one non-recurrent episode of infection with Salmonella in 16 years of follow-up arguing against a secondary effect. Consistently, it was recently demonstrated, employing an IL12RB2 knockout mice model, that lack of IL12 signaling predisposes to spontaneous lymphoproliferation, autoimmunity and B-cell lymphoma.[36] This phenotype was not caused by infections, because the mice were kept under pathogen-free conditions. However, infections are not an uncommon finding in ALPS patients especially after splenectomy[6] and infections by *Salmonella spp*. and *Klebsiella pneumoniae* have also been observed in a FasL-deficient patient.[39] Another case of ALPS-like syndrome with mycobacterial infection was reported.[43] Interestingly, Guerra *et al.* demonstrated that IL12-mediated upregulation of FasL and CD40L was involved in the control of mycobacterium tuberculosis growth by activated NK cells.[44] Deficiency in both IL12 and FasL expression can, therefore, contribute to susceptibility to infection. Previous analysis of patients with *IL12RB1* mutations demonstrated deficient IL12/JAK/STAT signaling (as could be confirmed for the patient analyzed here), lack of resulting IFNγ production by T and NK cells as well as deficient IL23 signaling and low levels of IL17-producing T cells.[45] A similar phenotype is described for targeted *IL12RB1* knockout mice.[46] In humans the clinical penetrance of *IL12RB1* mutations varies.

Heterozygous human carriers appear clinically normal with normal IL12/IL23 signaling and IFNγ production.[34] In two individuals of the analyzed family DNT cell numbers were slightly increased indicating possible effects of the heterozygous mutation on ALPS characteristics. We also detected increased numbers of DNT cells (14% of gated $CD3^+$ cells) in a patient with a complete loss of IFNγR2 expression; however, in this case it could not be ruled out that the increase was a response to frequent and recurrent infections (*data not shown*).

Our study demonstrates for the first time that loss of IL12RB1 expression in a patient leads to reduced upregu-

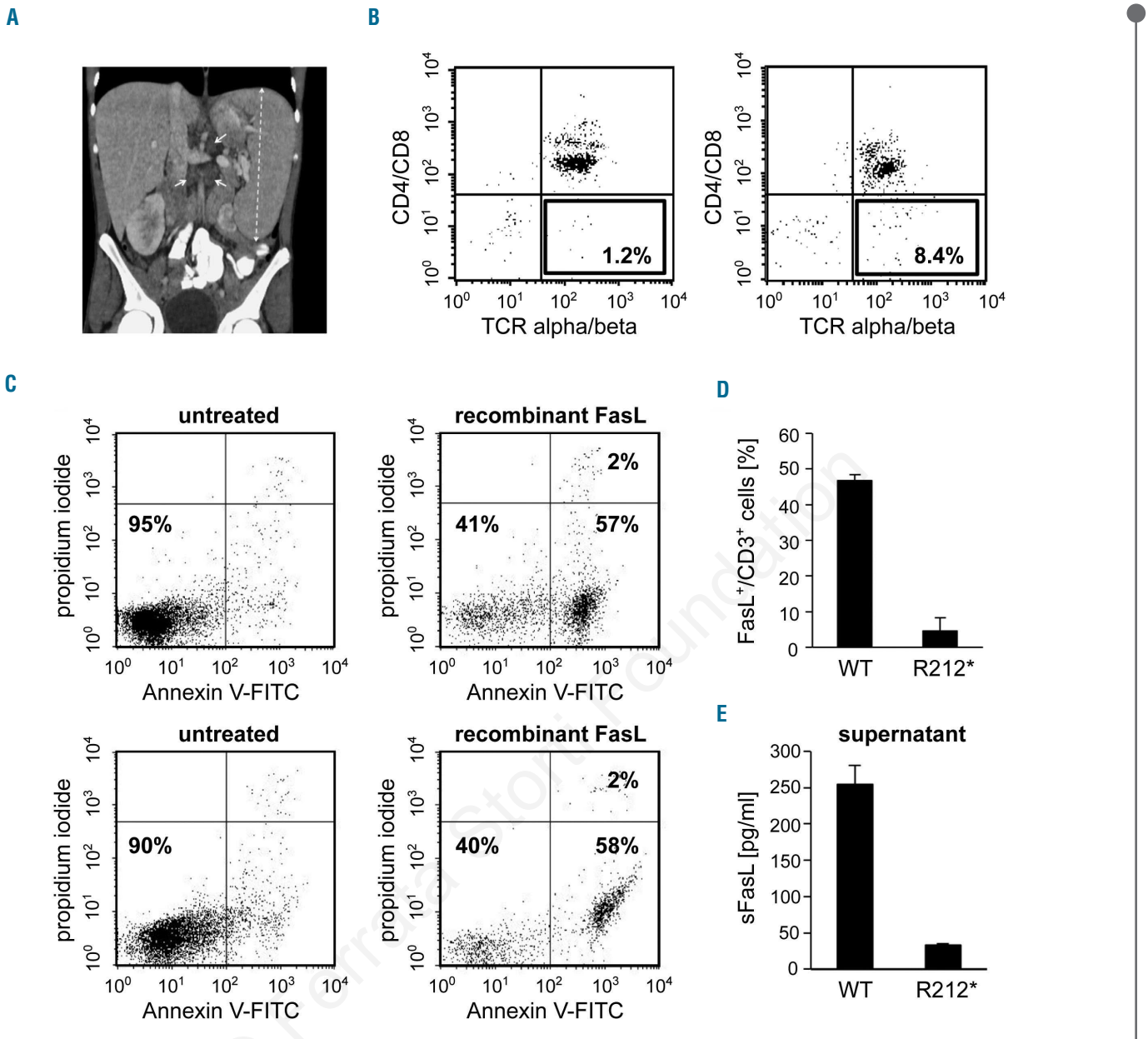

Figure 4. The IL12RB1-deficient patient presented with classical clinical ALPS symptoms. (A) Computed tomography showing the patient's splenomegaly and lymphadenopathy. Coronal reformatted image of the abdomen reveals significant splenomegaly (bidirectional dashed arrow) and enlarged retroperitoneal lymph nodes (arrows). (B) Elevated numbers of DNT cells (TCRα/β^+, $CD4^-$, $CD8^-$) in the peripheral blood of the patient (*right panel*, lower right quadrant) compared to a healthy control *(left panel)*. $CD3^+$ cells are shown. DNT cells are indicated in the rectangles. (C) Cultured healthy and patient's primary T cells undergo apoptosis after stimulation with recombinant FasL indicating a defect upstream of the Fas receptor. T cells were activated as described in Figure 1A. Apoptosis was induced by incubation with 100 ng/mL recombinant FasL for 16 h. Apoptosis was measured as described in Figure 1C. Apototic cells are presented in the lower right quadrant. *Left panel*: untreated, *right panel*: treated cells, *upper panels*: healthy wild-type control cells, *lower panels*: patient's cells. (D) Cell surface expression of membrane-bound FasL is lower in the patient's lymphocytes. Cells were activated by phytohemagglutinin (PHA) and IL2 (as described in Figure 1A) and FasL surface staining of $CD3^+$ T cells measured by flow cytometry on day 4. (E) Soluble FasL (sFasL) concentrations were measured by enzyme-linked immunosorbent assay. sFasL levels are lower in the supernatant of patient's cells after 4 days of PHA/IL2 treatment compared to levels from control cells. (B-E) Results of representative experiments repeated at least three times. Mean values and standard deviations of duplicates are shown in (D, E). Similar results were obtained using samples from at least three independent wild-type controls.

Table 2. Immunophenotyping of the patient and the heterozygous mutation carriers.

Parameter	Father	Mother	Sibling	Sibling	Patient	Normal values
DNT cells [$CD3^+$]	1.04%	**2.76%**	**3.6%**	1.74%	**8-27%**	<2.5%
$B220^+$ cells [DNT]	40%	24.11%	12.79%	5.26%	**92%**	<60%
$CD25^+$/HLA-DR^+ ratio	**0.98**	3.9	1.2	4.7	**0.58**	>1
$CD27^+$ cells [$CD19^+$]	18.65%	33%	**12.47%**	**12.5%**	**12%**	>15%

(The gated populations are indicated in brackets. Values deviating from normal values are presented in bold.)

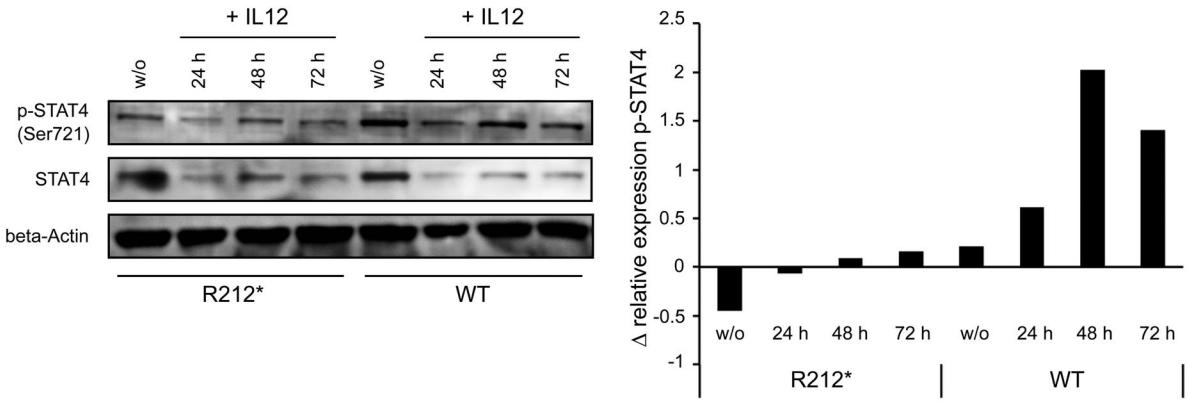

Figure 5. IL12-mediated signaling is disturbed in the patient's cells. *Left panel:* Phosphorylation of STAT4 in response to IL12 stimulation is abrogated in the patient. T cells of the patient and a healthy control were activated with phytohemagglutinin and IL2 as described in Figure 1A. Cell lysates were prepared after 24, 48 and 72 h of incubation with 200 ng/mL IL12 and untreated controls. Western blotting was carried out employing specific antibodies against total STAT4, phosphorylated STAT4 (serine 721) and β-actin as a loading control. *Right panel:* Densitometric measurement of signals derived by the western blot in the left panel carried out on a LAS-3000 equipped with LAS-3000 Image Reader software (Fujifilm, Düsseldorf, Germany). The difference of relative arbitrary units of pSTAT4 expression related to STAT4 expression is shown. The β-actin control was used to compensate differences due to loading. A representative result of two independent experiments is shown.

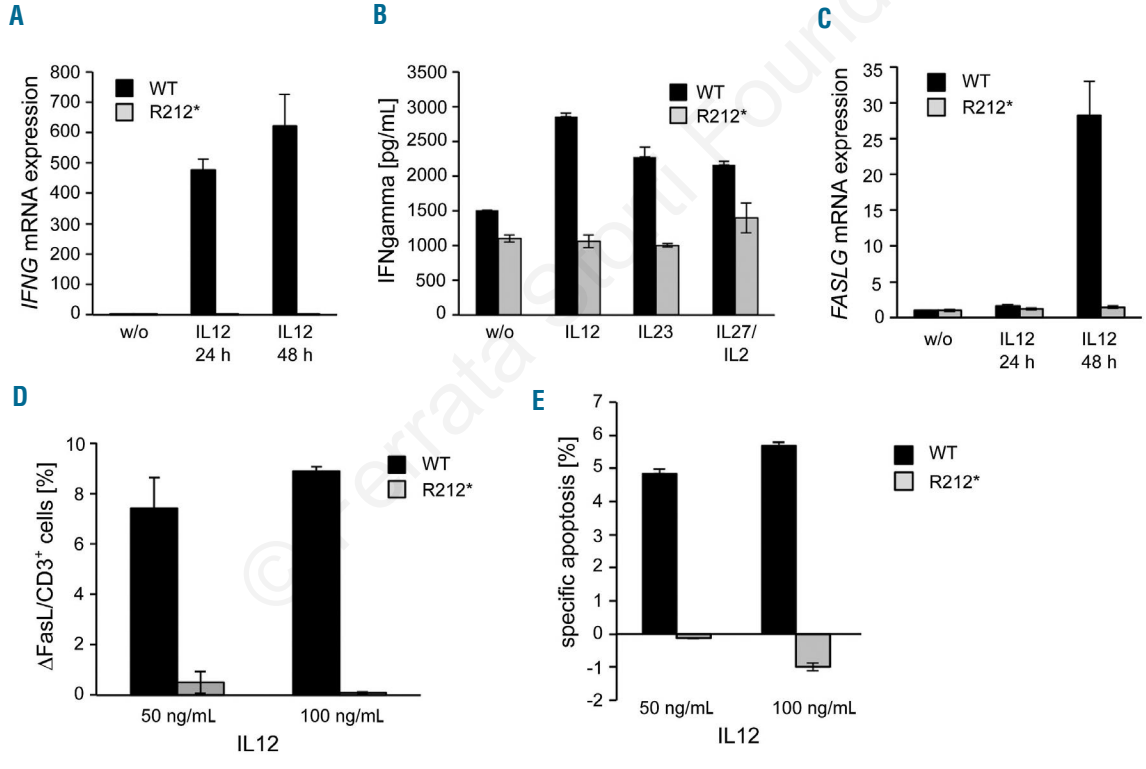

Figure 6. IL12-mediated signaling and apoptosis are disturbed in the patient's cells. (A) Primary T cells of the patient and a healthy control were activated by phytohemagglutinin (PHA) and IL2 treatment and incubated with or without IL12 (100 ng/mL) for 24 or 48 h, respectively. RNA was then extracted and *IFNG* mRNA expression was measured by real-time polymerase chain reaction. The fold change compared to untreated controls is shown. *GAPDH* expression was used as an internal standard. (B) IFNγ production after treatment of immortalized T cells with 100 ng/mL IL12, IL23 or IL2/IL27, for 2 days was measured by enzyme-linked immunosorbent assay. IFNγ production in response to IL12 or IL23 is lacking in homozygous *IL12RB1* c.698G>A, p.R212* mutated cells in contrast to control cells. IL2/IL27 treatment induces a slight increase in IFNγ production in both wild-type and *IL12RB1* mutated T cells. (C) Primary T cells of the patient and a healthy control were activated by PHA/IL2 treatment and incubated with or without IL12 (100 ng/mL) for 2 days. RNA was then extracted and *FASLG* mRNA expression was measured by real-time polymerase chain reaction (fold-change compared to untreated control is presented). (D) IL12-induced upregulation of FasL expression was measured in T cells by flow cytometry after activation with PHA/IL2, and stimulation with IL12 (50 or 100 ng/mL, respectively) for a further 3 days. T cells of the patient lacked upregulation of FasL protein expression in response to IL12 compared to a healthy control. (E) Primary T cells derived from peripheral blood of the patient and a healthy control were activated and expanded by PHA/IL2 treatment and incubated with 50 or 100 ng/mL IL12, respectively, for 3 days. Apoptosis was measured as described in Figure 1C. The bar diagram represents apoptosis specifically induced by IL12 treatment compared to no treatment. (A-E) Results of representative experiments repeated at least three times. (A, C) Present mean values and standard deviation of triplicate experiments, (B, D, E) of duplicate experiments. Similar results were obtained using at least three wild-type controls.

lation of FasL and loss of the apoptotic response of T cells to prolonged treatment with IL12. In addition, the general level of FasL expression in activated T cells and the level of secreted FasL in the plasma or cell culture supernatant were much lower in the patient than in controls. Lower levels of FasL expression in the patient are probably attributable to a lack of IFNγ, because transcription of the *FASLG* promoter is positively regulated by the interferon-regulatory factors IRF-1 and IRF-2.[47] Consistently, it has been reported that mononuclear cells from *IL12RB1*-deficient patients produce significantly lower amounts of IFNγ in response to mitogens or antigens[32,33] and low IFNγ production is a common trait of all reported knockout mice models that are deficient in *IL12RB1*, *IL12RB2* or *IL12* (p40 or p35).[48]

IL12 is known as a factor that can stimulate growth and function of T cells and the differentiation of naive T cells into Th1 cells. However, prolonged stimulation of T cells with IL12 leads to apoptosis and stimulation of T cells with IL12 during activation enhances the tendency to undergo Fas-mediated activation induced cell death due to upregulation of FasL and downregulation of the inhibitor FLIPs.[27] Therefore, similar to defective Fas/FasL signaling, absence of IL12 signaling could lead to decreased death of T cells and accumulation of autoreactive T cells. In addition, it has been shown that low levels of IL12 drive the differentiation of activated T cells to long-lived self-renewing memory $CD8^+$ T cells rather than to short-lived effector cells when acute infections resolve.[49] This is dependent on IL12-controlled expression of T-bet and reflected in the phenotype of T-bet knockout mice.[49,50]

In conclusion, our results identify *IL12RB1* as a new gene and IL12 signaling as a new pathway that may underlie the pathogenesis of ALPS-like disease due to their regulatory role in FasL expression. Our data suggest that mutations in *IL12RB1* may lead to different clinical phenotypes, including ALPS-like disease and Mendelian susceptibility to mycobacterial diseases. Knowledge of the genetic defect underlying an ALPS-like phenotype may thus have important implications for the choice of treatment options.

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Authorship and Disclosures

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