Mutations of *NKFB1* and *STAT3* in the pathogenesis of autoimmune lymphoproliferative syndrome like disorders

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Cyrill Schipp
aus Düsseldorf

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aus der Klinik für Kinder-Onkologie, -Hämatologie und Klinische Immunologie

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Referent:

Prof. Dr. Arndt Borkhardt

Klinik für Kinder-Onkologie, -Hämatologie und Klinische Immunologie

Universitätsklinikum Düsseldorf

Korreferent:

Prof. Dr. Holger Gohlke

Institut für Pharmazeutische und Medizinische Chemie

Heinrich-Heine-Universität Düsseldorf

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Summary

The autoimmune lymphoproliferative syndrome (ALPS) is a lymphoproliferative disease caused by defects in FAS mediated apoptosis. Symptoms include lymphadenopathy, hepatosplenomegaly, autoimmunity and a characteristic increase of double negative T-cells (DNTs; TCRαβ^{+} CD3^{+} CD4^{-} CD8^{-}). Most patients harbor mutations in *FAS, FASLG*, or *CASP10*. However, in roughly 20% of patients no genetic lesion in these genes can be found. In order to improve diagnosis and to provide individualized therapeutic options, knowledge of the underlying genetic cause is essential. To identify novel disease causing mutations in ALPS patients we collected a cohort of 30 children with ALPS-like disease of unknown genetic cause. The DNA of patients and their families was analyzed employing whole exome sequencing. Candidate mutations were functionally validated with regard to their pathogenicity.

In the here presented studies we characterized activating mutations in signal transducer and activator of transcription 3 (*STAT3*) and truncating mutations in nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1)*.

In two unrelated patients with ALPS like symptoms we found two *de novo* germline mutations in *STAT3* (c.G833A, p.R278H; c.T1181C, p.M394T). In luciferase reporter assays mutated proteins proved to be hyper-active both under baseline conditions and after IL-6 stimulation. This finding could be confirmed by immunoblot showing increased STAT3 phosphorylation and by upregulation of STAT3 target genes in qRT-PCR experiments. Patient derived lymphocytes showed resistance to various apoptotic stimuli that could be rescued in R278H mutated cells by treatment with the small molecule inhibitor of STAT3 S3I-201. Further analysis revealed that the apoptotic resistance was caused by a STAT3 mediated disruption of the balance between pro- and anti-apoptotic BCL-2 family proteins. We showed that compared to healthy controls application of the BCL-2 inhibitor ABT-737 in a nanomolar range was able to induce apoptosis significantly more effective in patient derived cells due to overexpression of BCL-2. Summarized we showed that activating germline mutations of *STAT3* can mimic ALPS by dysregulating the balance of BCL-2 family proteins and can potentially be treated with BCL-2 inhibitors.

Two other unrelated patients with ALPS like symptoms harbored truncating germline mutations in *NFKB1* (c.A137del, p.I47YfsX2; c.C469T, p.R157X). While the p.I47YfsX2 mutation was acquired *de novo* in the patient, the p.R157X mutation was segregated from the father to the patient and two siblings. However, only the patient was symptomatic while the father and the siblings just had a mild subclinical hypogammaglobulinemia. Both

mutations caused haploinsufficiency of the two gene products p50 and p105. This resulted in dysregulation of genes involved in the canonical NF-κB pathway under baseline conditions. Mutated cells also showed aberrant response to stimulation of the canonical NF-κB pathway with LPS, TNFα, or IL-1β. Strikingly, both patients lacked specific antibodies and did not show immunization after vaccination. Summarized we showed that deleterious mutations in *NFKB1* can lead to a phenotype that partly overlaps with ALPS. Patients had disrupted canonical NF-κB signaling and were deficient for specific antibodies. For the first time specific antibody deficiency was linked to a genetic cause by this study.

Taken together these studies extend the genetic variations found in patients with ALPS like disorders. All four patients presented, among other, with ALPS like symptoms including lymphadenopathy, hepatosplenomegaly, autoimmunity and elevated levels of DNTs. In the future screening for mutations in ALPS like patients should also include *STAT3* and *NFKB1*. In patients with activating *STAT3* mutations individualized therapeutic options may include the BCL-2 inhibitor ABT-199. In patients with deleterious *NFKB1* mutations therapy should exclude NF-κB inhibitors like cyclosporine A which is sometimes used to treat cytopenias in ALPS patients.

Zusammenfassung

Das autoimmune lymphoproliferative Syndrom ist eine lymphoproliferative Erkrankung, die durch einen Defekt des FAS induzierten Apoptose Signalwegs ausgelöst wird. Die klassischen Symptome sind Lymphadenopathie, Hepatosplenomegalie, Autoimmunität und eine charakteristische Erhöhung von doppelt negativen T-Zellen (DNTs; $TCR\alpha\beta^{+}$ $CD3^{+}$ $CD4^{-}$ $CD8^{-}$). Die meisten Patienten haben Mutationen in *FAS, FASLG,* oder *CASP10*. In ungefähr 20% aller Patienten ist die genetische Ursache bisher allerdings unklar. Zur Verbesserung der Diagnosestellung und zur Bereitstellung individueller Therapieoptionen ist jedoch eine genaue genetische Charakterisierung erforderlich. Um neue, krankheitsauslösende genetische Veränderungen in ALPS-Patienten zu finden haben wir eine Kohorte von 30 Kindern mit ALPS-ähnlichen Symptomen und unbekannter genetischer Ursache gesammelt. Die DNA der Patienten und ihrer Familien wurde mittels *whole exome sequencing* analysiert und auf krankheitsauslösende Veränderungen untersucht. Die so gefundenen Kandidaten wurden auf ihre Pathogenität untersucht.

In den hier präsentierten Studien charakterisierten wir aktivierende Mutationen in *signal transducer and activator of transcription 3* (*STAT3*) und trunkierende Mutationen in *nuclear factor of kappa light polypeptide gene enhancer in B-cells 1* (*NFKB1)*.

In zwei nicht verwandten Patienten mit ALPS-ähnlichen Symptomen haben wir zwei *de novo* Keimbahnmutationen in *STAT3* gefunden (c.G833A, p.R278H; c.T1181C, p.M394T). In Luciferase-Reporter Experimenten zeigten die mutierten Proteine eine Hyperaktivierung in unstimulierten wie auch in IL-6 stimulierten Zellen. Dieses Ergebnis konnte durch den Nachweis von erhöhter STAT3 Phosphorylierung im Immunoblot und durch eine erhöhte Expression von STAT3 Zielgenen in qRT-PCR Experimenten bestätigt werden. Lymphozyten der Patienten waren resistent gegenüber verschiedenen apoptotischen Stimuli. Diese Resistenz konnte in Zellen mit der R278H Mutation durch den STAT3 Inhibitor S3I-201 überwunden werden. Weitere Analysen offenbarten ein gestörtes Verhältnis zwischen pro- und anti-apoptotischen Proteinen der BCL-2 Familie als Ursache der Apoptose Resistenz. Wir zeigten, dass Patientenzellen, die bedingt durch STAT3 Hyperaktivität BCL-2 überexprimieren, empfindlicher für Apoptose nach Behandlung mit dem BCL-2 Inhibitor ABT-737 im nanomolaren Bereich waren. Zusammenfassend konnten wir aktivierende Keimbahn *STAT3* Mutationen als Auslöser ALPS ähnlicher Symptome durch Dysregulation der BCL-2 Proteinfamilie identifizieren. Betroffene Patienten könnten von einer Behandlung mit BCL-2 Inhibitoren profitieren.

Zwei weitere nicht verwandte Patienten wiesen trunkierende Keimbahnmutationen in *NFKB1* auf (c.A137del, p.I47YfsX2; c.C469T, p.R157X). Während die p.I47YfsX2 Mutation in der einen Patientin *de novo* auftrat, wurde die p.R157X Mutation in der anderen Familie vom Vater an die Patientin und zwei weitere Kinder vererbt. Bis auf diese Patientin sind die anderen Mutationsträger in der Familie jedoch gesund und weisen nur eine leichte subklinische Hypogammaglobulinämie auf. Beide Mutationen führten zur Haploinsuffizienz der Genprodukte p50 und p105. Als Folge waren viele Gene, die im kanonischen NF-κB Signalweg eine Rolle spielen schon im unstimulierten Zustand dysreguliert. Nach Stimulation des kanonischen NF-κB Signalwegs mit LPS, TNFα oder IL-1β zeigten Zellen beider Patienten ein anormales Verhalten. Auffallend war in beiden Patienten ein Abhanden sein von spezifischen Antikörpern und eine erfolglose Immunisierung nach Impfungen. Zusammenfassend konnten wir zeigen dass deletierende Mutationen in *NFKB1* zu einem Phänotyp führen der mit ALPS überlappt. Diese Patienten hatten einen gestörten kanonischen NF-κB Signalweg und hatten eine spezifische Antikörper Defizienz. In dieser Arbeit konnte zum ersten Mal eine genetische Veränderung mit spezifischer Antikörper Defizienz in Zusammenhang gebracht werden.

Zusammen betrachtet erweitert diese Studie die Vielfalt der genetischen Veränderungen in Patienten mit ALPS-ähnlichen Symptomen. Alle vier beschriebenen Patienten präsentierten sich unter anderem mit dem klassischen ALPS-Phänotyp, bestehend aus Lymphadenopathie, Hepatosplenomegalie, Autoimmunität und einer erhöhten Zahl von DNTs. Im zukünftigen Screening von ALPS-Patienten sollten die Gene *STAT3* und *NFKB1* eingeschlossen werden. Dadurch könnten zum einen Patienten mit aktivierenden *STAT3* Mutationen mit dem BCL-2 Inhibitor ABT-199 behandelt werden. Zum anderen sollten Patienten mit trunkierenden *NFKB1* Mutationen nicht mit NF-κB Inhibitoren behandelt werden. Dazu gehört Cyclosporin A, ein Mittel das gelegentlich zur Behandlung von Zytopenien in ALPS-Patienten angewandt wird.

I Introduction

1.1 Lymphocyte apoptosis

Proper lymphocyte homeostasis is a cornerstone of a functional immune system. While generation and maturation of the correct amount of immune cells is obviously of utmost importance, apoptotic removal of autoreactive or activated lymphocytes is necessary for a healthy immune system. In general apoptosis can be triggered either by receptor-ligand interactions at the cell surface (extrinsic pathway) or by the release of mitochondrial cytochrome c (intrinsic pathway) leading to processing and activation of caspases and their downstream targets (Figure 1) [1].

A pivotal role in the extrinsic pathway of lymphocyte apoptosis is played by the death receptor FAS, a member of the tumor necrosis factor receptor superfamily. FAS is highly expressed on activated lymphocytes and part of the negative feedback mechanism called propriocidal regulation which eliminates excess effector cells during the immune response [2]. Binding of trimerized FAS ligand (FASLG) by the trimerized FAS receptor leads to the recruitment of the adaptor protein FADD followed by procaspases 8 and 10 (CASP8/10). The resulting Death-inducing signaling complex (DISC) incites CASP8/10 maturation which leads to effector caspase activations (caspase cascade). This process culminates in protein proteolysis and the death of the cell [1]. However, in most cells the DISC signal needs to be amplified via the intrinsic pathway, therefore a crosstalk between the extrinsic and intrinsic apoptosis pathway is essential. Crucial for the crosstalk is the small pro apoptotic BCL-2 family protein BID. After cleavage by CASP8 or CASP10, cleaved BID (tBID) can interact with other BCL-2 family proteins, whereupon the intrinsic pathway is activated [3].

The intrinsic apoptosis pathway is controlled by proteins of the BCL-2 family which is characterized by four conserved sequence motifs called the BCL-2 homology domains (BH1-4). The BCL-2 family proteins can be separated into three distinct subgroups (i.e., BH3-only, pro-apoptotic, anti-apoptotic) based on functional and structural properties. The pro-apoptotic proteins (e.g. BAX, BAK) can form oligomers within the outer mitochondrial membrane which leads to formation of a pore and subsequent cytochrome c release from the mitochondrion. This instigates assembly of the apoptosome in the cytoplasm resulting in the caspase cascade and cell death. The anti-apoptotic proteins (e.g. BCL-2, BCL-XL, MCL-1) keep the pro-apoptotic ones in check by binding their BH3 domain and therefore inhibiting their oligomerization. BH3 only proteins (e.g. BIM, BAD, NOXA) act as a pathway-specific sensor for various stimuli. They compete with the

BH3 binding grove of anti-apoptotic BCL-2 family proteins and thereby promote oligomerization of the pro-apoptotic proteins [4].

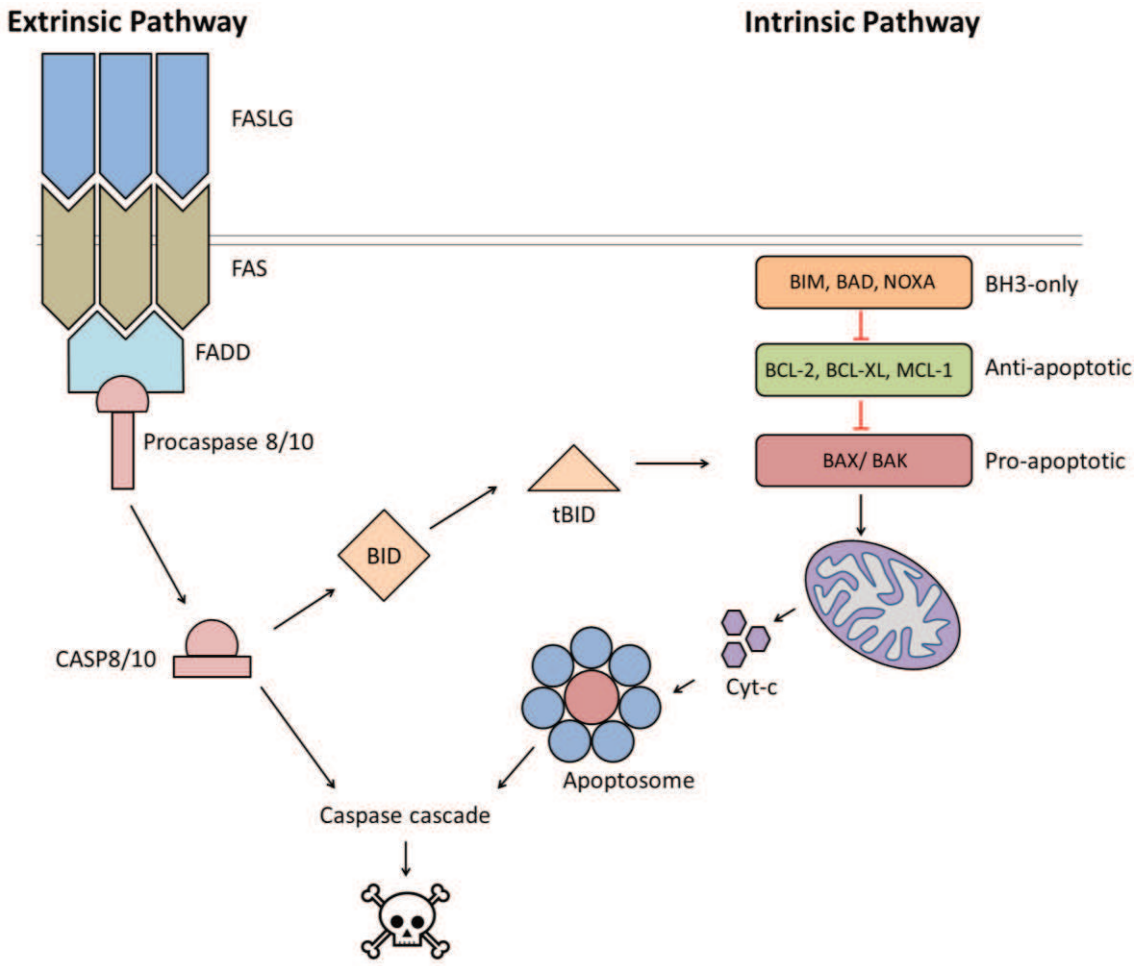

Figure 1: The intrinsic and the FAS mediated extrinsic apoptosis pathway. Binding of FASLG by the FAS homotrimer leads to a complex of FAS, FADD and CASP8 and/or CASP10 (death inducing signaling complex, DISC) which activates downstream effector caspases resulting in programmed cell death. The intrinsic apoptosis pathway is characterized by a balance of pro- and anti-apoptotic BCL-2 family proteins. This balance can be tipped by binding of BH3-only proteins to the anti-apoptotic family members causing oligomerization of the pro-apoptotic proteins in the outer mitochondrial membrane and subsequent cytochrome c release. This induces apoptosome assembly and programmed cell death. Crosstalk between the extrinsic and intrinsic pathway is mediated by tBID, the product of BID cleavage by CASP8/10. In turn tBID can interact with pro-apoptotic BCL-2 family proteins and induce outer mitochondrial membrane permeabilization via BAX/BAK.

1.2 Autoimmune lymphoproliferative syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a lymphoproliferative disease typically diagnosed in early childhood. Canale and Smith first described five patients with lymphadenopathy, hepatosplenomegaly and autoimmune cytopenia in 1967 [5]. In 1992 Sneller et al. recognized that two mouse strains known as *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) exhibit a similar phenotype as ALPS patients. Intriguingly, they showed that patients as well as mice feature an increase of a rare T-lymphocyte subpopulation, so called "double negative T-cells (DNT)", which express the T-cell receptor and CD3 but neither of the differentiation markers CD4 or CD8 ($TCR\alpha/\beta^+$, $CD3^+$, $CD4^-$, $CD8^-$) [6]. Later homozygous mutations in *Fas* and *Faslg* were found to be responsible for the phenotype in *lpr* and *gld* mouse strains [7,8]. Subsequently, different groups were able to show heterozygous and homozygous germline *FAS* mutations in ALPS patients thus connecting ALPS with a defect in FAS mediated apoptosis [9,10].

Heterozygous mutated FAS can exert a dominant negative effect on homo-trimer formation in such a way that statistically only 1 in 8 trimer consists of three wildtype FAS proteins while the remaining trimers contain at least one mutated protein leading to a reduced or absent ability to receive or transduce apoptotic signals. These mutations are generally inherited in an autosomal-dominant manner, however with incomplete penetrance [11]. Penetrance is strongly associated with the mutation locus. While the penetrance of mutations in the intracellular domain of FAS is very high (>90%), penetrance of mutations in the extracellular domain is substantially lower (<30%) [12,13]. Interestingly, recent studies showed that somatic acquisition of further somatic *FAS* mutations or loss of heterozygosity was mandatory for disease onset in families with non-penetrant *FAS* mutations [14,15]. These somatically acquired mutations either affect the intracellular domain or the other, second allele. Consistently, it has become increasingly clear, that next to germline *FAS* mutations the second most common genetic cause for ALPS are somatic *FAS* mutations [16]. These can only be detected in a small subset of cells, most often DNTs. Nevertheless, clinical manifestations are similar to germline mutations.

Apart from *FAS* mutations other members of the FAS-signaling pathway have been found mutated in ALPS patients. Homozygous as well as heterozygous mutations of *FASLG* have been described in few patients [17-19]. These mutations were either missense mutations altering the extracellular domain and exerting a dominant negative effect during trimer formation similar to heterozygous FAS mutations or resulted in protein truncation and abrogation of FAS death receptor signaling. Also, heterozygous mutations in *CASP10* were found in a small number of ALPS patients [20,21]. These mutations reduced the catalytic

activity and thereby disrupted downstream signaling. Since CASP10 is part of different DISCs, it was shown that *CASP10* mutations not only affected FAS signaling but other death receptor pathways like TRAIL as well. Mutations in FADD and CASP8 have been found in patients with a phenotype resembling ALPS, however these disorders have distinctive features and were therefore classified separately. They will be discussed in the segment about ALPS related disorders.

In 2009 the classification of ALPS was revised by the First International ALPS Workshop (Table 1) [22]. At that time about 20% of patients diagnosed with ALPS did not have mutations in the known ALPS genes (*FAS*, *FASLG*, *CASP10*). With the advances in high-throughput DNA sequencing technology a number of new monogenetic variations in ALPS patients have been discovered in recent years. Since these are not classified as ALPS subtypes, these too will be covered in the part about ALPS related disorders.

Table 1: Revised classification of ALPS [1,22]

Nomenclature	Gene	Description	Frequency
ALPS-FAS	*FAS*	Patients fulfill ALPS diagnostic criteria and have germline mutations in *FAS*	~ 70%
ALPS-sFAS	*FAS*	Patients fulfill ALPS diagnostic criteria and have somatic mutations in *FAS*	~ 10%
ALPS-FASLG	*FASLG*	Patients fulfill ALPS diagnostic criteria and have germline mutations in *FASLG*	~ 1%
ALPS-CASP10	*CASP10*	Patients fulfill ALPS diagnostic criteria and have germline mutations in *CASP10*	~ 2%
ALPS-U	Unknown	Patients fulfill ALPS diagnostic criteria; however genetic defect is undetermined (no *FAS*, *FASLG* or *CASP10*)	~ 20%

1.2.1 Clinical and laboratory features

ALPS is a genetic disorder with the median age of 24 month for the first onset, however with an increasing awareness also adult patients are now diagnosed more frequently with ALPS [23]. The main clinical manifestation is chronic lymphadenopathy with no infectious or malignant cause which can be found in more than 95% of patients. Additionally 95% of patients experience splenomegaly and 72% present with hepatomegaly. Beside the enlargement of primary or secondary lymphatic organs, autoimmune cytopenias are the second most common clinical manifestation. Roughly 70% of patients suffer from autoimmune hemolytic anemia (AIHA), autoimmune neutropenia or immune-mediated thrombocytopenia (ITP), which are frequently accompanied by multiple autoantibodies and a positive Coombs test [1]. The development of other autoimmune disorders like uveitis,

hepatitis, glomerulonephritis, encephalitis and myelitis has been described in some ALPS patients during their long term follow-up [24-27]. Since apoptosis is critical in tumor surveillance with FAS as a putative tumor suppressor, it is not surprising that ALPS patients have an increased risk of hematologic malignancies. The risk to develop Hodgkin lymphoma was reported to be 50 times higher in ALPS patients compared to the general population and 14 times higher for non-Hodgkin lymphoma, even in asymptomatic mutation carriers [28]. Lymphoma surveillance should therefore be of utmost importance in ALPS patients and their families.

The most significant laboratory characteristic of ALPS patients is the elevation of DNT numbers (TCRα/β$^+$, CD3$^+$, CD4$^-$, CD8$^-$ T-cells). While in the peripheral blood of healthy individuals the DNT population is less than 2.5% of CD3$^+$ T-cells (or less than 1.5% of total lymphocytes), in ALPS patients DNTs can reach up to 20% of T-cells because of dysfunctional apoptotic clearance [22]. Origin and function of DNT cells is still not entirely clear. For a long time the main hypothesis of DNT origin argued that they derive from exhausted CD4$^+$ or CD8$^+$ T-cells after stimulation [29,30], however recently the idea that these are an independent subset of regulatory T-cells deriving from the thymus has gained support [31]. Also, the paradigm that DNTs are only a phenomenon but not functionally involved in disease progression has lately been overturned. It was found that DNTs are highly expressing Interleukin-10 (IL-10). IL-10 is strongly linked to autoimmunity and elevated in plasma of ALPS patients (see below) [32,33]. Interestingly, DNTs in *lpr* mice could selectively be eliminated by inhibition of the Notch signaling pathway which also resulted in decreased autoimmunity in this model [34].

The characteristic defect in FAS mediated apoptosis can be assessed by *in vitro* apoptosis assays. Activated peripheral blood mononuclear cells (PBMC) are treated with FAS activating antibodies or recombinant FASLG and the rate of apoptosis is compared to cells of similarly treated healthy individuals. This assay however can only give a meaningful result in ALPS-FAS and ALPS-CASP10 patients, since patients with ALPS-sFAS or ALPS-FASLG will not show a phenotypical change *in vitro*. Because of that, apoptosis assays are not performed regularly to determine an ALPS diagnosis anymore. Instead, a variety of biomarkers have been defined that can predict ALPS with high likelihood. It was shown that ALPS patients have elevated serum levels of the cytokines TNFα, IL-10 and IL-18 as well as soluble FASLG (sFASLG) and vitamin B_{12} [35,36]. Elevation of these biomarkers is highest in patients with ALPS-FAS or ALPS-sFAS but in patients with other ALPS subtypes they are still moderately raised and useful for diagnosis. Often ALPS patients present with hypergammaglobulinemia indicated by elevated IgG, IgA or IgM levels, yet few patients

have hypogammaglobulinemia or even common variable immunodeficiency (CVID) [37,38]. Histopathological characteristics of affected lymph nodes in patients include paracortical expansions due to DNT infiltration accompanied by follicular hyperplasia and polycloncal plasmacytosis [23].

In light of these new insights into clinical and laboratory ALPS manifestations, the diagnostic criteria got revised by the First International ALPS Workshop in 2009 (Table 2). They now consist of two required criteria as well as primary- and secondary accessory criteria. A definitive diagnosis is based on the presence of both required criteria plus one primary accessory criterion. A probable diagnosis is based on both required criteria plus one secondary accessory criterion [22]. The required criteria consist of chronic nonmalignant, noninfectious lymphadenopathy or splenomegaly and pathological elevated DNTs. Primary accessory criteria include a defect in lymphocyte apoptosis and somatic or germline mutations in the classical ALPS genes (FAS, FASLG, CASP10), while secondary accessory criteria take into account elevated serum biomarkers (IL-10, IL-18, sFASLG, vitamin B_{12}), presence of autoimmune cytopenias, immunohistological findings and related family history.

Table 2: Revised diagnostic criteria for ALPS [22]

Required
1. Chronic (>6 month), nonmalignant, noninfectious lymphadenopathy or splenomegaly or both
2. Elevated $CD3^+$ $TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ cells (>1.5% total lymphocytes;>2.5 of $CD3^+$ lymphocytes) in the setting of normal or elevates lymphocytes counts
Accessory
Primary
1. Defective lymphocyte apoptosis
2. Somatic or germline mutation in *FAS*, *FASLG* or *CASP10*
Secondary
1. Elevated plasma levels of sFASLG (>200 pg/ml) or IL-10 (>20 pg/mL) or IL-18 (>500 pg/ml) or vitamin B_{12} (>1500 ng/l)
2. Typical immunohistological findings
3. Autoimmune cytopenias (AIHA, ITP or neutropenia) **and** polyclonal hypergammaglobulinemia
4. Family history of nonmalignant, noninfectious lymphoproliferation with or without autoimmunity

1.2.2 Therapy and prognosis

Therapy of ALPS is mainly focused on symptomatic treatment to maintain or improve the patient's quality of life. Lymphadenopathy and splenomegaly caused by lymphoproliferation can be relieved by the mTOR-inhibitor rapamycin (sirolimus) which proved to normalize DNT levels and reduce lymph node along with spleen size in ALPS patients [39]. For some time splenectomy was considered a viable alternative in patients with splenomegaly but recent studies showed that, because of complications in form of multiple episodes of live threatening sepses and absence of long term improvement of other symptoms, it should be avoided [1,37]. Autoimmune cytopenias are generally only treated short term during critical periods by various treatment strategies like corticosteroids, mycophenolate mofetil (MMF), intravenous immunoglobulin G or rituximab since long time administration shows many side effects [1]. Interestingly, long time rapamycin medication also reduced the autoimmune phenotype with few side effects, however, careful monitoring for its toxicities is required [39]. Due to its beneficial effects on lymphoproliferation as well as autoimmunity, rapamycin is becoming the treatment of choice in ALPS patients especially in those with more severe phenotypes [40,41]. Close clinical observation with computed tomography (CT) or positron emission tomography (PET) in regular intervals is needed to monitor for lymphomas in the enlarged lymph nodes.

The only curing therapy for ALPS so far is hematopoietic stem cell transplantation (HSCT) which has been reported to be successful in ALPS patients with lymphoma or a severe phenotype due to homozygous FAS mutations [42,43]. Patients with less urgent indication have been transplanted with variable results. Due to the fact that applicable related donors often harbor the disease causing mutation themselves despite being asymptomatic, unrelated donor derived HSCT would be required in most cases. But HSCT has a too high mortality rate to be considered for ALPS patients without a severe phenotype. Only if a patient's lymphoproliferation is likely to require life-long potent immunosuppression with its own adverse effects, HSCT should be taken into account [1].

The prognosis for ALPS patients in general is very good since lymphadenopathy and autoimmunity decrease in most patients during puberty. With proper lymphoma surveillance they are expected to live a normal life span with few clinical complications. Out of 347 patients monitored in two recent studies only 19 died of disease or treatment related causes, twelve of which due to infections after splenectomy. The other deaths were caused by malignancies (4), anemia (2) and stroke (1) [1,37]. With splenectomy resulting in the highest cause of death in these studies it should strongly be discouraged in ALPS

patients. Instead spleen guards should be considered in patients with massive splenomegaly to reduce the risk of splenic rupture [44].

1.2.3 Autoimmune lymphoproliferative syndrome related disorders

ALPS related disorders are characterized by an ALPS similar phenotype with some distinctive features that let them be categorized separately. These patients often present with recurrent infections, multi-organ lymphocytic infiltrations or hypogammaglobolinemia while partly fulfilling ALPS diagnostic criteria with lymphadenopathy, splenomegaly, elevated DNTs or autoimmuntiy in such a way that they often get initially diagnosed with ALPS. A selection of these disorders is presented below (Table 3).

Interestingly, defects in the DISC proteins CASP8 and FADD lead to defective lymphocyte apoptosis but to a distinct phenotype compared to ALPS-FAS or ALPS-CASP10. Patients with homozygous germline *CASP8* mutations have been reported with a defect in antigen activation of T-, B- and NK-cells and multi organ lymphocytic infiltrations in addition to ALPS symptoms [45-47]. The lymphocyte activation defect is due to the role of CASP8 in NF-κB activation after antigen receptor stimulation [48]. Prior to the revision of the ALPS classification these patients were classified as an ALPS subtype but because of the distinct phenotypic features this immune defect is now termed as caspase-8 deficiency state (CEDS). Patients with deficiency of FADD present without lymphadenopathy or splenomegaly yet DNT levels are elevated as well as ALPS biomarkers. These patients suffer from functional hyposplenism with pneumococcal and viral infections [49]. The FADD phenotype can likewise be attributed to the multifunctional role of FADD in the immune system. It is for example also involved in NF-κB activation and plays a central role in the antiviral innate immune response [48,50].

Patients with somatic activating mutations of *NRAS* or *KRAS* in the hematopoietic linage were previously classified as ALPS patients. This disorder was reclassified as RAS-associated autoimmune leukoproliferative disease (RALD). It is set apart from ALPS by persistent granulocytosis, monocytosis and IL-2 independent proliferation of T-cells [51-54]. Gain-of-function mutations in *NRAS* or *KRAS* result in a permanently activated RAS-ERK signaling pathway which promotes cell cycle progression and leads to partial apoptotic resistance by degradation of the BH3 only protein BIM [52,55]. Other ALPS related disorders that were classified by the International ALPS Workshop are Dianzani autoimmune lymphoproliferative disease (DALD) for which no genetic cause is known until now except

for a weak link to *UNC13D* mutations [56], and X-linked lymphoproliferative syndrome (XLP1) which is caused by mutations in *SH2D1A* [57,58].

Mutations in *PRKCD*, *LRBA* or *CTLA4* have been found in families with immune dysregulation and different phenotypes. Patients with lymphadenopathy, hepatosplenomegaly, autoimmunity and EBV infections were found with a deficiency in the protein kinase PKCδ (*PRKCD* gene). This kinase plays a critical role in regulation of lymphocyte proliferation, apoptosis and differentiation. Bizarrely, patients were reported to have either B-cell lymphoproliferation or B-cell deficiency. Remarkably, patient derived B-cells showed a striking oversecretion of IL-10 *in vitro* which could be responsible for the ALPS like phenotype [59,60]. Deficiency of LRBA was found in patients with lymphoproliferation and autoimmunity. These patients have a very variable phenotype but around 20% are initially diagnosed with ALPS. Frequently observed symptoms include lymphadenopathy, splenomegaly, autoimmunity as well as recurrent infections and hypogammaglobulinemia [61]. LRBA is involved in endocytosis of ligand activated receptors and thus an integral part of signal transduction. Primary T-cells of ALPS patients with LRBA deficiency were less responsive to apoptosis induced by FASLG but simultaneously more sensitive to apoptosis stimulated by serum deprivation or staurosporine [62]. It was also shown that defects in LRBA decreased the sensitivity of tumor cell apoptosis [63].

Recently one patient carrying a homozygous mutation in the IL-12 and IL-23 receptor subunit *IL12RB1* was described. The patient presented with a classical ALPS phenotype. It was shown that deficiency of IL12RB1 leads to perturbed FASLG expression and thereby resembling ALPS-FASLG. Furthermore IL-12 and IL-23 induced IFN-γ expression and secretion was absent in primary T-cells [64]. In the last two years numerous patients with activating germline *STAT3* mutations were found with a phenotype resembling ALPS. Lymphadenopathy, hepatosplenomegaly, autoimmunity and elevated DNTs were accompanied by respiratory tract infections and hypogammaglobulinemia. However, the pathophysiological mechanism remained unclear (see 1.3.2) [65-67].

Table 3: ALPS related disorders [22] Abbreviations: CEDS: caspase-8 deficiency state; RALD: RAS-associated autoimmune leukoproliferative disease; DALD: Dianzani autoimmune lymphoproliferative disease; XLP1: X-linked lymphoproliferative syndrome; GOF: gain-of-function

Nomenclature	Gene	Description
CEDS	*CASP8*	Lymphadenopathy/Splenomegaly, elevated DNTs, recurrent infections, defective lymphocyte activation, lymphocytic infiltrations, homozygous germline *CASP8* mutations [45-47]
RALD	*NRAS, KRAS*	Lymphadenopathy/Splenomegaly, autoimmunity, granulocytosis and monocytosis, T-cells IL-2 independent, activating somatic *NRAS* or *KRAS* mutations [51-54]
DALD	---	Lymphadenopathy/Splenomegaly, autoimmunity, defective *in vitro* FAS mediated apoptosis [68]
XLP1	*SH2D1A*	Fulminant EBV infection, hypogammaglobulinemia, lymphoma, defective T-cell receptor mediated apoptosis, homozygous germline *SH2D1A* mutations [57,58]
FADD deficiency	*FADD*	Functional hyposplenism, pneumococcal and viral infections, elevated DNT, homozygous germline *FADD* mutations [49]
PKCδ deficiency	*PRKCD*	Lymphadenopathy/Hepatosplenomegaly, EBV infection, autoimmunity, homozygous germline *PRKCD* mutations [59,60]
LRBA deficiency	*LRBA*	Lymphadenopathy/Splenomegaly, autoimmunity, recurrent infections, hypogammaglobulinemia, chronic lung disease, homozygous germline *LRBA* mutations [61-63,69,70]
CTLA4 haploinsufficiency	*CTLA4*	Lymphadenopathy/Splenomegaly, autoimmunity, elevated DNTs, hypogammaglobulinemia, lymphocytic infiltrations, enteropathy, germline CTLA4 mutations [71-73]
IL12RB1 deficiency	*IL12RB1*	Lymphadenopathy/Hepatosplenomegaly, autoimmunity, highly elevated DNTs, homozygous germline *IL12RB1* mutations [64]
STAT3 GOF	*STAT3*	Lymphadenopathy/Hepatosplenomegaly, autoimmunity, elevated DNTs, respiratory tract infections, hypogammaglobulinemia, activating germline *STAT3* mutations [65-67]

1.3 The JAK-STAT signaling pathway

Communication between cells by cytokines and surface receptors is central in lymphocyte homeostasis and the initiation of the immune response. One of the best understood means for signal transduction from the cell surface to transcriptional activation of downstream genes is the JAK-STAT signaling pathway (Figure 2). The signal transduction structure is conserved from invertebrates to mammals [74]. Upon dimerization of a cytokine receptor by ligand engagement, receptor bound Janus kinases (JAK) phosphorylate each other and the receptor, thereby allowing signal transducers and activators of transcription (STAT) proteins to bind to the receptor where they are also phosphorylated and thereby activated by JAKs. After activation, STATs form homo dimers which translocate into the nucleus and regulate target gene transcription. Genes that are positively regulated by STATs include suppressors of cytokine signaling (SOCS) which inhibit JAK function and receptor phosphorylation resulting in a negative feedback loop [75,76]. Negative regulation of this pathway can also be mediated by the protein inhibitors of activated STAT (PIAS) protein family. They can directly interact with STAT homodimers and negatively influence nuclear translocation and transcriptional activation of their target genes [77].

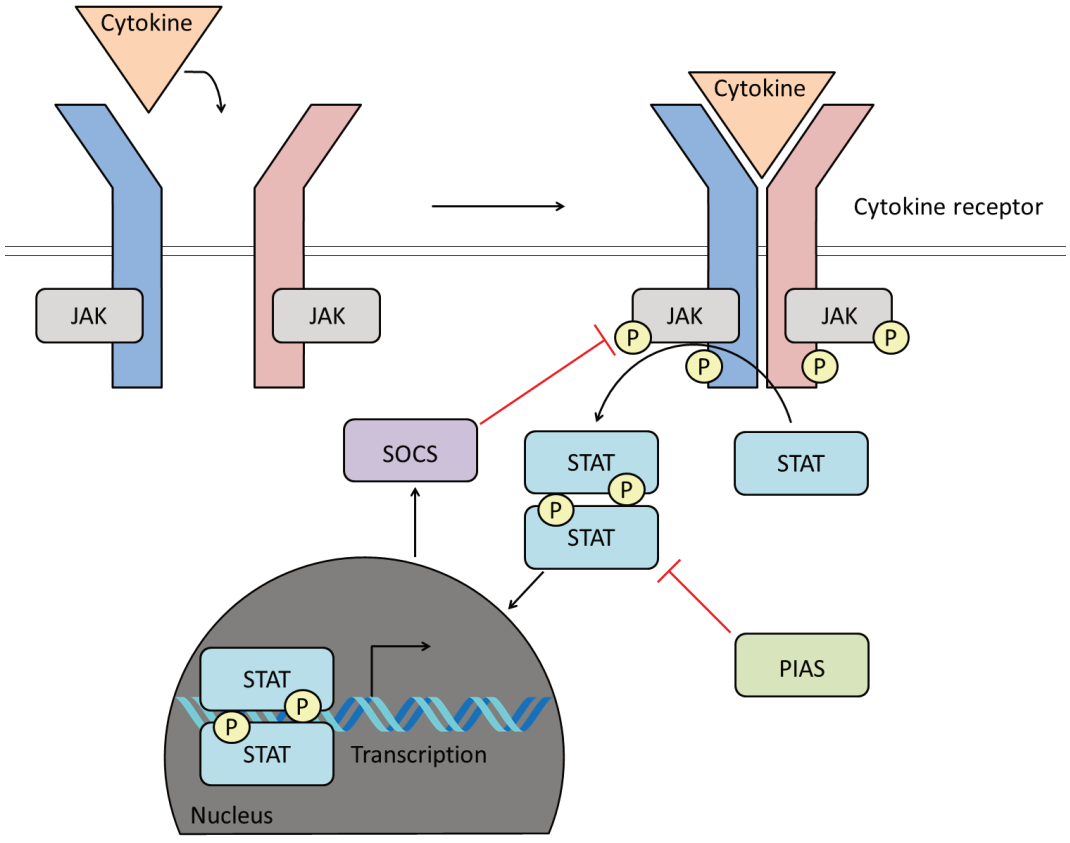

Figure 2: The JAK-STAT signaling pathway. Ligand binding by the cytokine receptor leads to dimerization followed by activation of receptor associated JAKs. STATs can then bind to the receptor where they are phosphorylated by JAKs and form homodimers. These can translocate into the nucleus where they bind DNA and regulate target gene transcription. STAT mediated SOCS expression results in a negative feedback loop. Other negative regulators of JAK-STAT signaling are proteins of the PIAS family.

1.3.1 The key players

The JAK-STAT pathway is involved in signal transduction by many different cytokine receptors, including receptors for interferons (IFN), interleukins (IL) and other growth factors [75]. Each receptor subunit is selectively associated with one JAK family member (JAK1, JAK2, JAK3 and TYK2) in such a way that every receptor preferentially uses a specific or combination of JAKs. Each JAK contains a conserved C-terminal kinase domain and an adjacent catalytically inactive pseudo kinase domain which is important for proper kinase domain function while the N-terminal FERM and SH2 domain are responsible for cytokine receptor binding (Figure 3). Although it has been shown that each JAK can bind to a wide variety of cytokine receptors which gives rise to the assumption of interchangeability of different JAKs, knockout studies in mice show that receptors have a clear preference for the specific JAK they utilize [78]. For example the IFN-γ receptor uses the combination of JAK1 and JAK2, whereas the IL-2 receptor uses the combination of JAK1 and JAK3 [79]. Generally JAK3 and TYK2 are involved in the immune response while JAK1 and JAK2 have broader functions in homeostasis, proliferation and even neural development. This is emphasized by the fact that deletion of either *Jak1* or *Jak2* is embryonically lethal in mice but *Jak3* or *Tyk2* deletion is not [80]. Surprisingly, JAK1 and JAK2 are able to translocate into the nucleus and have a direct role in gene expression and cell growth, however the mechanism behind this is still poorly understood [81].

The STAT family consists of seven proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) with an N-terminal coiled coil domain, a DNA-binding domain as well as a C-terminal SH2 and a transactivation domain (Figure 3). After receptor phosphorylation the SH2 domain docks to the receptor and is phosphorylated on a conserved tyrosine residue by JAKs. By binding of this phosphorylated tyrosine the SH2 domains of two STAT molecules then initiate dimerization which is followed by nuclear translocation. In the nucleus the DNA-binding domain binds to target gene promotors and the transactivation domain can regulate their transcription [76,77,82]. The coiled coil domain is essential for stabilizing protein-protein interactions of STATs. For example it was shown to be required for SH2 receptor binding [83].

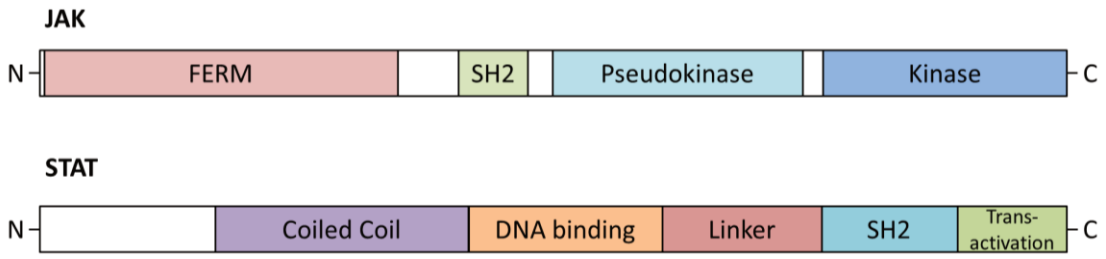

Figure 3: Schematic domain structure of JAK and STAT. JAK activity is mediated by its kinase domain, which requires the pseudokinase domain for proper function. The FERM and SH2 domains are responsible for receptor association. The DNA-binding, SH2 and transactivation domain of different STATs are highly homologous. The SH2 domain mediates receptor binding and dimerization, while the coiled coil domain plays a role in stabilizing protein-protein interactions.

Similar to JAKs, each cytokine receptor prefers specific STAT proteins with little interchangeability. Knockout studies in mice showed that absence of one STAT can lead to increased phosphorylation of another, with massive changes in transcriptional profile compared to the wildtype [84,85]. These studies showed aberrant receptor signaling in all STAT deficiencies except for *Stat3* which was not included because it is embryonically lethal. STATs regulate thousands of genes as well as non-coding RNAs [75]. Target gene expression profiles are not only determined by the involved STAT but also by the cytokine receptor as well as the cell type. STAT6 signaling by IL-4 for instance induces a completely different set of genes in macrophages compared to T-cells, yet IFN-γ signaling via STAT1 induces the expression of a similar group regardless of the cell type [79,86]. Another more complex example is the poorly understood difference between the IL-6 and IL-10 response in macrophages which induce transcription of a very distinct set of genes even though both use JAK1 and STAT3 for signal transduction [79].

The best understood regulators of the JAK-STAT pathway are SOCS and PIAS proteins. The SOCS family has eight members (CIS, SOCS1-7). They are induced by the JAK-STAT pathway and form a negative feedback loop by inhibiting further JAK kinase activity [87]. Additionally, SOCS can induce ubiquitination of JAKs and cytokine receptors for rapid proteasomal degradation [88,89]. Apart from the negative feedback loop, SOCS play a very important role in the crosstalk between different JAK-STAT mediated cytokine pathways, for example in monocytes interferon signaling can inhibit IL-4 signaling by induction of SOCS1 [90]. While SOCS inhibition acts on JAK and cytokine receptors, proteins of the PIAS family (PIAS1, PIAS3, PIASX, PIASY) target STATs directly by binding dimeric STATs and inhibiting their transcriptional activation [91,92]. A couple of different mechanisms have been indicated for PIAS function. PIAS are able to abolish the DNA-binding ability of some STAT dimers [92,93] and recruit HDACs to silence STAT target genes [91,94]. Also, PIAS have SUMO

ligase activity, yet the effect of STAT sumoylation is discussed controversially. Some reports are showing a decrease in DNA-binding activity of STAT1-SUMO while others show conflicting data on whether sumoylation of STAT1 is essential for PIAS mediated inhibition or not [95-97].

Since cytokine signaling is essential in blood cell homeostasis, mutations in the JAK-STAT pathway have been linked to different diseases. On the one hand, somatic activating mutations of JAKs and STATs resulting in a constitutive active pathway are found in many different hematologic malignancies of the myeloid as well as the lymphoid linage. On the other hand, loss of function mutations lead to reduced or aberrant cytokine signaling and are linked to different immunodeficiency disorders [80].

1.3.2 STAT3 in health and disease

First discovered as a member of the epidermal growth factor (EGF) and IL-6 pathway, STAT3 is a key transcription factor in innate and adaptive immunity [98]. The number of pathways identified to activate STAT3 have risen since then and include numerous cytokine and growth factor receptors as well as intracellular tyrosine kinases [99]. Phosphorylation on a conserved tyrosine residue (P-Tyr705) of STAT3 leads to homodimerization mediated by the SH2 domain and transcriptional activation of target genes. Additionally, C-terminal serine phosphorylation (P-Ser727) plays a role in transcriptional regulation by STAT3 [100,101].

The transcriptional program regulated by STAT3 involves genes that mediate proliferation, differentiation and survival [102-104]. STAT3 induces its own expression forming a positive feedback loop but at the same time starts a negative feedback loop by activating the expression of SOCS3 [105,106]. The role of the positive autoregulatory loop despite the upstream JAK inhibition by SOCS3 became clear when regulatory involvement of unphosphorylated monomeric STAT3 (uSTAT3) was found [107,108]. It can bind to similar target genes as phosphorylated STAT3 but also interacts with the NF-κB pathway thereby regulating a number of different genes compared to activated dimers [109]. However, the transcriptional program remains similar. For example, pro survival signaling through uSTAT3 works by suppression of pro-apoptotic genes while STAT3 induces expression of anti-apoptotic BCL-2 family proteins like BCL-2, BCL-XL and MCL-1 [110-113]. Interestingly, STAT3 also represses expression of the FAS receptor, therefore increasing resistance to apoptosis even further [114]. Apart from the transcriptional activities of STAT3 it also plays a role as a negative regulator for autophagy or in the mitochondrion where it regulates oxidative phosphorylation and generation of reactive oxygen species [115-117].

The most compelling way to understand STAT3 function in the immune system is to analyze patients with autosomal dominant Hyper-IgE syndrome (AD-HIES). These patients harbor heterozygous loss-of-function *STAT3* (STAT3-LOF) mutations resulting statistically in only 25% functional STAT3 dimers and therefore markedly reduced cytokine signaling through STAT3. More than 50 STAT3-LOF mutations have been described until now with the majority disrupting the DNA-binding, SH2, or transactivation domain [118]. The most common clinical characteristics include increased levels of IgE, eczema, as well as recurring bacterial and fungal infections of the skin, lungs, or upper airways [119,120]. Serum concentration of IgE can be 100 fold higher than in healthy individuals (>1000 IU/ml) frequently accompanied by eosinophilia. These patients have a defect in naïve $CD4^+$ T-cell differentiation with the biggest effect on Th17 cell generation [121]. Th17 cells are known to play a central role in immunity against bacterial and fungal infections but are nearly absent in AD-HIES patients [120,122]. Furthermore, patients have impaired B-cell function with a defective T-cell dependent, antigen specific antibody response [123,124].

To investigate the pathogenesis of AD-HIES a mouse was designed that heterozygously expresses a STAT3-LOF variant. Interestingly, in this mouse model hematopoietic stem cell transplantation was not enough to restore the inflammatory and immune response, indicating an important function of STAT3 in non-hematologic cells for the immune system [125].

Recently, it has become clear that not only STAT3-LOF but also *STAT3* gain-of-function (STAT3-GOF) mutations have a pathophysiological effect in hematologic diseases. While somatic STAT3-GOF mutations are associated with malignancies, germline mutations are connected to a lymphoproliferative phenotype with autoimmunity, resembling ALPS.

Somatic STAT3-GOF was first described with low frequency in inflammatory hepatocellular adenomas, a benign liver tumor predominantly found in women [126]. The first connection to a hematologic malignancy was made when STAT3-GOF mutations were found in up to 70% of large granular lymphocytic (LGL) leukemia [120,127-131]. LGL leukemia is a chronic clonal lymphoproliferative disorder of abnormally large granular lymphocytes that derive from either T- or NK-cells. Patients frequently experience autoimmune conditions like rheumatoid arthritis and immune mediated cytopenias. In other malignant entities STAT3 mutations are rare, however few reports showed STAT3-GOF in diffuse large B-cell lymphoma (DLBCL) [132,133]. DLBCL is the most common type of adult non-Hodgkin lymphoma and highly activated JAK-STAT signaling is common in patients and DLBCL cell lines [134]. Remarkably, most somatic STAT3-GOF mutations are located in the

SH2 domain (Figure 4, marked in red). Constitutive activation in these cases is likely caused by the stabilization of STAT3 homodimers resulting in uncontrolled proliferation and an increased resistance to apoptosis in affected cells thus driving clonal expansion. It was shown that STAT3-GOF mutations in the SH2 domain lead to increased STAT3 phosphorylation and thereby increased target gene expression in cells both unstimulated and even more so after IL-6 stimulation [128].

In the search for a *de novo* mutation in a pediatric patient with early-onset multi-organ autoimmunity, a germline STAT3-GOF mutation was first described by Flanagan et al.. Further analysis of their cohort revealed five patients with four different STAT3 mutations [65]. In quick succession publications of similar patients reported a total of 19 patients with germline STAT3-GOF mutations [65-67]. These patients were mainly characterized by short stature, autoimmunity, and recurrent respiratory tract infections. Autoimmune manifestations included type 1 diabetes, autoimmune enteropathy, AIHA, ITP, and autoimmune neutropenia. Some patients were initially diagnosed with ALPS. These patients additionally presented with lymphadenopathy, hepatosplenomegaly, elevated DNTs, and resistance to FAS mediated apoptosis without mutations in FAS related genes [67]. In contrast to somatic mutations, germline STAT3-GOF mutations do not cluster mainly in the SH2 domain but are mostly spread in the DNA-binding and the SH2 domain (Figure 4, marked in green). The reported mutations showed increased transcriptional activity in luciferase reporter assays but no constitutive phosphorylation when overexpressed in reporter cells [65-67]. Yet, in bone marrow biopsies of two patients abnormally high numbers of P-Tyr705 positive lymphocytes were detected [66]. Also PBMCs of another patient showed delayed de-phosphorylation of STAT3 after stimulation with IL-6. This patient received monoclonal IL-6 antibodies (tocilizumab) treatment and showed clinical improvement of his polyarthritis and scleroderma within months [67]. Two patients underwent HSCT with mixed results. One died from severe graft-versus-host disease 138 days after transplantation, while the other experienced complete remission of the autoimmune disease and improved growth [67]. Otherwise treatment was mostly symptomatic to improve the autoimmunity and fight infections. Patients stay in poor condition and have to receive regular immunoglobulin replacement therapy or even weekly red blood cell transfusion [66].

Targeted treatment of hyperactive STAT3 is so far only applicable via upstream inhibition of cytokine receptors or JAKs since there are no clinically approved STAT3 inhibitors. However, upstream inhibition always gives rise to side effects because STAT3 is not the only downstream substrate in cytokine signaling and upstream inhibition does also not affect baseline hyperactivity of STAT3. There are only few STAT3 inhibitors currently tested in clinical trials of phase 1 thus application of these compounds is far away [135]. Therefore, a better understanding of the pathogenesis of STAT3-GOF induced diseases is necessary to identify new treatment options.

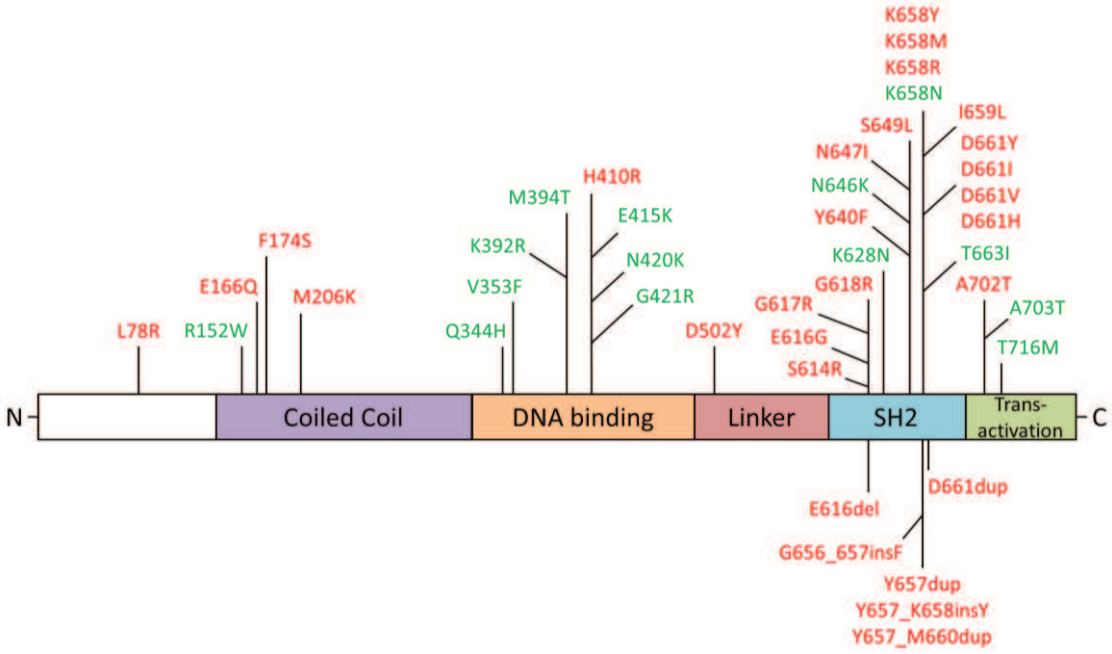

Figure 4: Schematic representation of reported gain-of-function mutations in STAT3. Mutations in green represent germline STAT3-GOF mutations, while somatic STAT3-GOF mutations found in malignancies are indicated in red. Mutations above the figure show missense mutations while mutations below show small insertions or deletions [65-67,120,126-133,136].

1.4 The NF-κB signaling pathway

First described as transcriptional activator for immunoglobulin kappa light-chain expression, another central signaling pathway in the development and function of the immune system is the NF-κB pathway [137]. It regulates numerous immunological functions, most notably inflammation, antigen response, and cytokine production [138]. There are two distinct ways for NF-κB activation, the canonical and the non-canonical pathway. While the non-canonical pathway predominantly activates the transcription factor dimer p52:RELB, the canonical activates a variety of transcription factor dimers via IκB degradation.

1.4.1 The canonical NF-κB pathway

The most studied receptors to activate canonical NF-κB signaling are the TNF-receptor (TNFR), Toll-like receptor (TLR), and IL-1 receptor (IL-1R). Upon receptor activation inhibitors of κB (IκB) signaling are phosphorylated by IκB kinases (IKK). IκBs undergo proteasomal degradation and release nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) transcription factors. These translocate into the nucleus where they regulate target gene transcription. Apart from the core components (NF-κB, IκB, IKK) the canonical NF-κB pathway relies on numerous other proteins like adaptor proteins (e.g., CARD9, CARD11), ubiquitin ligases (e.g., HOIL1, HOIP), or upstream protein kinases (e.g., PKC).

At the core of the NF-κB signaling pathway (Figure 5) are five transcription factors of the NF-κB protein family (p65, RELB, c-REL, p50 (*NFKB1*), p52 (*NFKB2*)). NF-κB proteins form homo- or heterodimers via interaction of their conserved Rel homology domains (RHD) which are essential not only for dimerization but also for DNA binding. NF-κB dimers bind to κB sites of target genes where they can upregulate or repress transcription. Transcriptional activation is mediated by a transactivation domain (TAD) which can only be found in p65, RELB and c-REL. The lack of a TAD in p50 results in an inhibitory effect of p50 homodimers by competing with TAD-containing dimers for κB binding sites. However, in combination with another NF-κB transcription factor p50 can also positively regulate gene transcription [139]. DNA binding studies showed that different dimers prefer slightly different DNA sequences indicating the complexity of the pathway [140]. Phosphorylation and other post-translational modifications of NF-κB proteins are important for activation and play a role in signaling pathway crosstalk. For instance, p65 phosphorylation increases its DNA-binding affinity and allows recruitment of different co-activators [141,142].

The transactivation activity of NF-κB proteins is inhibited by proteins of the IκB family (i.e., IκBα, IκBβ, IκBε, IκBζ, BCL-3, p105 (*NFKB1*), p100 (*NFKB2*)). They bind the RHD domain of NF-κB dimers with their ankyrin repeat (ANK) domain and keep them sequestered in the cytoplasm thereby inhibiting nuclear shuttling and DNA binding. Pathway activation leads to IκB phosphorylation on conserved serine residues. This phosphorylation induces polyubiquitination and subsequently proteasomal degradation of the IκB protein followed by nuclear translocation of the bound NF-κB dimer. Interestingly, the IκB proteins BCL-3 or IκBζ can also form a transcriptional activating complex with the p50 homodimer, however their role is not completely understood [143]. The IκB proteins p105 and p100 are the precursors of p50 and p52 respectively. N-terminal cleavage after phosphorylation releases p50 or p52, while the full length proteins contains a C-terminal ANK domain that binds NF-κB dimers [138]. In contrast to p100 which is only processed by activation of the non-canonical NF-κB pathway, p50 is constitutively produced resulting in a defined p105/p50 ratio [138].

IκB proteins are phosphorylated by a complex of two catalytically active IκB kinases (IKKα, IKKβ) and one regulatory subunit (IKKγ (NEMO)). The phosphorylation of IκB in the canonical pathway is performed by IKKβ after phosphorylation on two serine residues whereas IKKα is known to be essential for downstream signaling yet the exact role remains unclear [144-146]. Both kinases bind to IKKγ with their NEMO binding domain (NBD) which is critical since blocking of this interaction results in NF-κB inhibition [147]. Indispensable for the activation of NF-κB signaling is the ubiquitin binding ability of IKKγ. For example IL-1R signaling leads to poly-ubiquitination of receptor associated kinases. IKKγ binds to these ubiquitin chains allowing phosphorylation of IKKβ and subsequent NF-κB signaling. Loss of IKKγ ubiquitin binding capability abrogates IL-1R signaling [148].

With the importance of NF-κB signaling in the immune system, tight regulation of all components is crucial to keep the balance between immune response and autoimmunity. Similar to the JAK-STAT pathway, constitutive activation of the NF-κB pathway can be found in different malignancies. Increased cytokine release of the microenvironment during pathogenic inflammation is one way to achieve elevated NF-κB activation during tumor genesis. Thus, patients with chronic inflammatory disease have a higher risk for cancer [142,149]. Activating mutations in the NF-κB pathway are another way for the tumor to obtain hyper-activated NF-κB signaling. Activating NF-κB mutations are especially found in lymphomas. For example, LOF mutations in IκB proteins or GOF mutations in REL, as well as activating mutations in accessory proteins have been described in Hodgkin lymphoma and DLBCL [150-153]. Compared to activating mutations in malignancies,

immunodeficiencies due to monogenetic inactivating mutations in the NF-κB pathway are more frequent. These patients have a variable phenotype depending on the affected gene. However, commonly observed are recurring infections, autoinflammation, and defects in lymphocyte activation [154]. Well known are defects of IKKγ or IκBα resulting in impaired canonical pathway response in patients with ectodermal dysplasia with immunodeficiency. In these patients, mutated IKKγ disrupts assembly of the IKK complex, while IκBα mutations results in degradation resistance and therefore inhibits translocation of the NF-κB proteins to the nucleus [155,156].

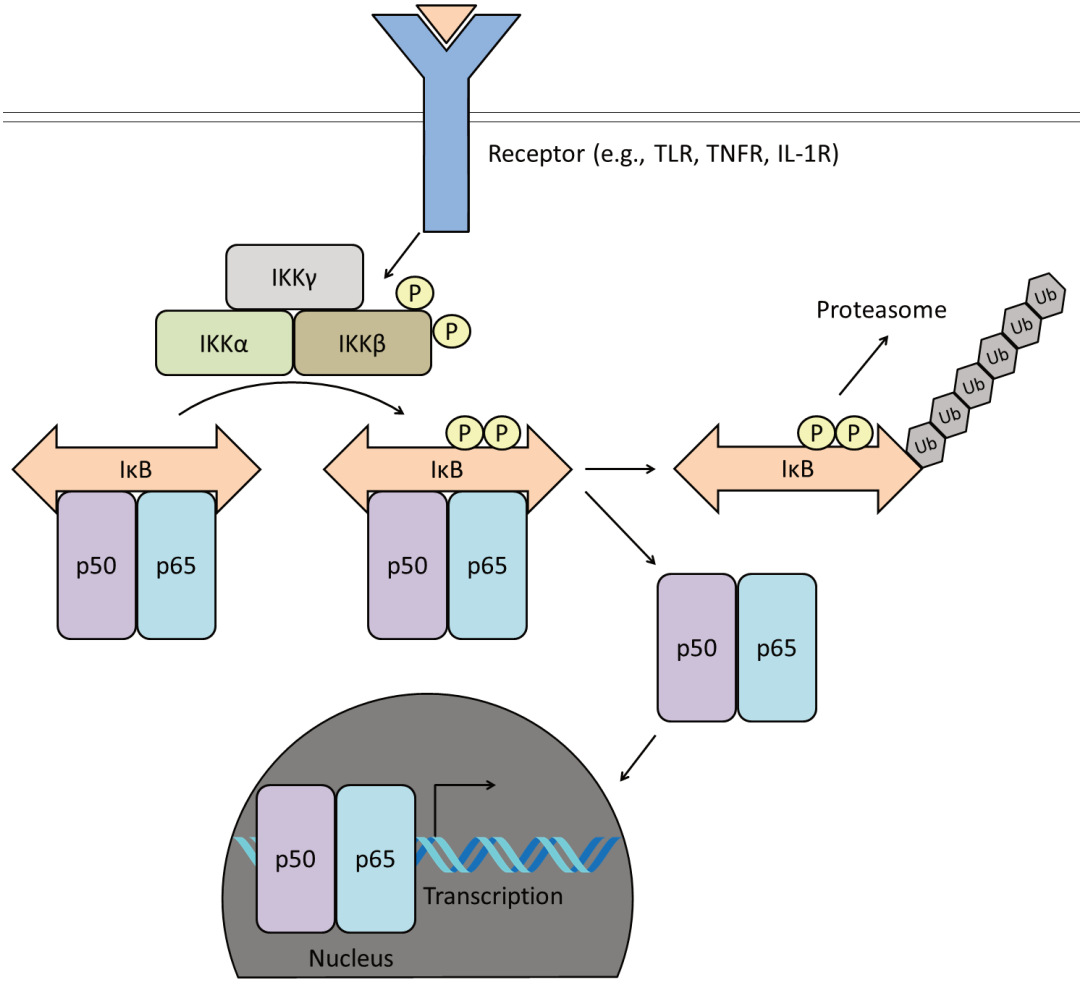

Figure 5: The canonical NF-κB signalling pathway. Ligand binding of numerous receptors (e.g., Toll-like receptors (TLR), TNF-receptors (TNFR), IL-1 receptor (IL-1R)) can activate the NF-κB pathway. Homo- or heterodimers of NF-κB transcription factors (p65, RELB, c-REL, p50 (NFKB1), p52 (NFKB2)) are sequestered in the cytosol by IκB proteins. After receptor activation, the IKK complex can phosphorylate IκB which leads to rapid proteasomal degradation and NF-κB translocation into the nucleus where they can regulate the transcription of target genes.

1.4.2 The *NFKB1* gene

The *NFKB1* gene encodes the IκB protein p105 which can be processed to the NF-κB protein p50 (Figure 6). Processing of p105 to p50 is regulated by a number of NF-κB dependent as well as independent mechanisms. On the one hand, p50 can be generated by proteasomal degradation during translation. This results in functional p50 prior to the completion of full-length p105 translation [157]. On the other hand, processing of p105 can be triggered by phosphorylation at serine residues in the PEST domain (S927, S932). Phosphorylated p105 recruits the ubiquitin ligase KPC1 which ubiquitinates p105 at the C-terminus leading to partial proteasomal degradation and release of p50 [158]. Yet, a translation and ubiquitination independent mechanism of p105 processing has also been described [159]. Essential for any kind of p105 processing is the glycine-rich region (GRR). Cells overexpressing GRR deletion constructs could not generate p50 [160]. Different reports attribute this to the GRR being either an initiation or stop signal for proteasomal cleavage [159,160]. Apart from its function as IκB protein, p105 has other regulatory purposes. Most notably, p105 together with p100 forms a high molecular mass inhibitory complex which is critical for the control of NF-κB signaling [161]. Also p105 was found to regulate the functions of proteins that are associated with the NF-κB pathway, like the anti-apoptotic protein c-FLIP or the mitogen associated kinase TPL2 [162,163].

Transcriptional regulation by p50 can either have an activating or an inhibitory effect on target genes depending on the dimer partner. In complex with a TAD-containing NF-κB protein (p65, RELB, c-REL) it exerts an activating effect on target gene transcription, while p50 homodimers are supposed to be transcriptional repressors [164]. Regulation of dimer formation is still poorly understood. A possible IκB function in dimer selection was postulated when IκBβ was found to promote p65 homodimerization [165]. Also different residues inside the RHD have been found to be essential for either p50 homodimerization or generation of p50:RELB heterodimer [166,167]. With its bifunctional role in target gene regulation p50 is an important factor to monitor the immune response and especially inflammation. Heterodimers can initiate the expression of pro-inflammatory cytokines to start the inflammation, while p50 homodimer prevent prolonged inflammation by competitively repressing the pro-inflammatory response. Additionally, p50 homodimers can interact directly with histone deacetylases (HDAC) and thereby inhibit transcription of pro-inflammatory genes in a non-competitive manner [168]. Furthermore, it was shown that p50 homodimers can interact with BCL-3 to induce expression of anti-inflammatory genes [169].

Studies in *Nfkb1* knockout mice (*Nfkb1*$^{-/-}$) helped to understand the role of p50 and p105 in the innate as well as the adaptive immune response. Defects of innate immunity include impaired myelopoeisis with reduced numbers of myeloid progenitors and defective TLR signaling of plasmacytoid dendritic cells [170,171]. In particular, macrophages of *Nfkb1*$^{-/-}$ mice showed an aberrant expression of genes induced by IFN signaling after TLR activation which was attributed to the loss of repressive p50 homodimers [164,172]. The effects of *Nfkb1* knockout on the adaptive immune response were even more pronounced. *Nfkb1*$^{-/-}$ mice showed B-cell defects in proliferation, survival and antibody production. They also had an increased mortality rate after infection with different bacterial pathogens [173]. Other studies in *Nfkb1*$^{-/-}$ mice showed defective proliferation and function in cytotoxic T-cells as well as T-helper cells after stimulation [174,175]. Another mouse model that lacks p105 but not p50 (*Nfkb1*$^{\Delta C/\Delta C}$) was designed to investigate the different roles of these two proteins. In contrast to *Nfkb1*$^{-/-}$ mice the *Nfkb1*$^{\Delta C/\Delta C}$ mice showed no negative effect on B-cell function, in fact proliferation and antibody production was slightly increased compared to wildtype animals. However, *Nfkb1*$^{\Delta C/\Delta C}$ mice did have abnormal activation and homeostasis of T-cells and were more susceptible to bacterial infections. Interestingly, *Nfkb1*$^{\Delta C/\Delta C}$ mice developed intestinal inflammations comparable to human patients with inflammatory bowel disease indicating an anti-inflammatory role of p105 [176,177].

A connection between *NFKB1* and human disease was first made when an *NFKB1* promotor polymorphism was associated with the inflammatory disease ulcerative colitis. It was shown that the polymorphism results in reduced expression of the *NFKB1* gene *in vitro* [178]. In 2015 Fliegauf et al. described for the first time mutations in *NFKB1* in human immunodeficiency patients [179]. They discovered two splicing and one frameshift mutation that segregated in an autosomal-dominant manner in three families with several cases of CVID. These mutations resulted in haploinsufficiency of p50 and p105. Strikingly, the described mutations did not show complete penetrance resulting in mutation carriers without any clinical symptoms. The clinical presentation of the patients was remarkably variable, yet most patients had hypogammaglobulinemia and recurring infections, especially of the respiratory tract. Other findings include autoimmune phenomena and lymphoma. However, compared to immunodeficient patients with mutations upstream in the signaling pathway of p50/p105 or *Nfkb1*$^{-/-}$ mice, no defects of the innate immune system were found [179].

The variable phenotype of patients with deleterious *NFKB1* mutations and the incomplete penetrance of these mutations give reason to assume that numerous CVID patients harbor *NFKB1* mutations. Analysis of more patients can give insight into disease pathogenesis and might improve therapeutic options.

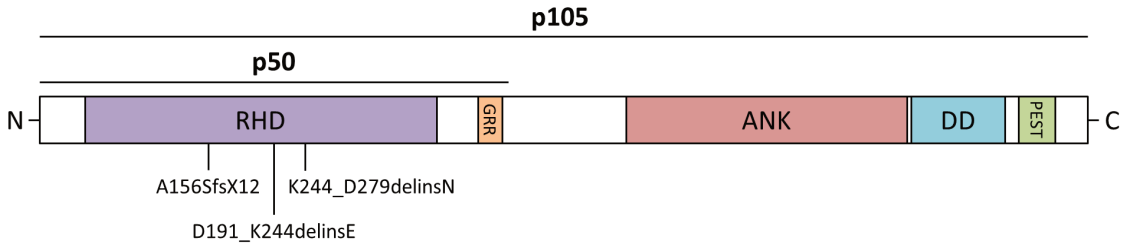

Figure 6: Schematic structure of the NFKB1 gene products. The *NFKB1* gene encodes the p105 protein and its processed form p50. Essential for this processing is the glycine-rich region (GRR) as well as phosphorylation of the PEST domain. p50 contains the Rel homology domain (RHD) that is conserved in all NF-κB proteins. In addition, p105 contains the typical IκB Ankyrin repeat domain (ANK) and a death domain (DD). Marked under the figure are reported deleterious mutations in patients with CVID [179].

II Scientific aims

The pathogenesis of ALPS is tightly linked to defects in lymphocyte apoptosis mediated by FAS signaling. Most patients have mutations in *FAS*, *FASLG*, or *CASP10.* However, around 20% of ALPS patients do not have a mutation in these classical ALPS genes. We collected a cohort of 30 children with ALPS like disease of unknown genetic cause and performed whole exome sequencing to find disease causing mutations in these patients. The aim of this thesis was to analyze the sequencing data for possible candidate mutations. The pathogenicity of these candidates in the immune system should be functionally validated in regard to the ALPS like phenotype.

Here, I am presenting two different projects that arose from four patients of this cohort of ALPS like patients. Of these four patients two harbored a germline mutation in *STAT3* and two harbored a germline *NFKB1* mutation. The functional validation for all candidates was performed with the following strategy:

1. Investigation of the mutations' effect on protein function.

2. Investigation of the mutations' effect on downstream signaling.

3. Impact of the mutation on FAS induced apoptosis and other apoptotic pathways.

4. Comparison of the clinical and laboratory phenotype to classical ALPS.

III Key Points

Following conclusions can be drawn from this work:

STAT3 project

1. Constitutively activating STAT3 mutations can cause typical ALPS symptoms including lymphadenopathy, increased number of DNTs and autoimmunity. Therefore, ALPS patients should also be analyzed for *STAT3* mutations in the future.
2. Patient derived cells are resistant to apoptosis induced by various stimuli. Apoptosis resistance is caused by dysregulation of BCL-2 family proteins resulting in a shift to an anti-apoptotic state. Additionally, a downregulation of FAS expression reinforces FASLG resistance.
3. Patient derived cells are susceptible to BH3 mimetics that target BCL-2 and induce apoptosis in BCL-2 dependent cells. This might be a possible treatment option in patients with activating *STAT3* mutations since clinically approved STAT3 inhibitors are lacking.

NFKB1 project

1. Deleterious *NFKB1* mutations result in p50/p105 haploinsufficiency and can cause typical ALPS symptoms including lymphadenopathy, increased number of DNTs and autoimmunity. In addition, patients lack specific antibodies after vaccination and can therefore not be immunized. Also patients show auto-inflammation of the lung.
2. Haploinsufficiency of p50/p105 causes dysregulation of NF-κB pathway members in immune cells resulting in aberrant downstream signaling after stimulation with canonical pathway activators (LPS, TNFα, IL-1β).
3. Penetrance of p50/p105 haploinsufficiency is not complete. However, healthy mutation carriers can show a mild subclinical hypogammaglobulinemia.

IV References

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V Manuscripts

5.1.1 *STAT3* gain-of-function mutations associated with autoimmune lymphoproliferative syndrome like disease deregulate lymphocyte apoptosis and can be targeted by BH3 mimetic compounds

Schafiq Nabhani[1]*, **Cyrill Schipp[1]***, Hagit Miskin[2§], Carina Levin[3§], Sergey Postovsky[4], Tal Dujovny[3], Ariel Koren[3], Dan Harlev[2], Anne-Marie Bis[1], Franziska Auer[1], Baerbel Keller[5], Klaus Warnatz[5], Michael Gombert[1], Sebastian Ginzel[1,6], Arndt Borkhardt[1], Polina Stepensky*[7], Ute Fischer*[1]

[1]Department of Pediatric Oncology, Hematology and Clinical Immunology, University Children's Hospital, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany
[2]Pediatric Hematology Unit, Shaare Zedek Medical Center, Jerusalem, Israel
[3]Pediatric Hematology Unit Emek Medical Center, Afula, Israel
[4]Department of Pediatric Oncology/Hematology Meyer Children's Hospital Rambam Health Care, Haifa, Israel
[5]Center for Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany
[6]Department of Computer Science, Bonn-Rhine-Sieg University of Applied Sciences, Sankt Augustin, Germany
[7]Department of Bone Marrow Transplantation, Hadassah Hebrew University Medical Center, Jerusalem, Israel

*SN and CS contributed equally as first authors, §HM and CL contributed equally as second authors, *PS and UF contributed equally as last authors

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Contributions of Cyrill Schipp (co-first author)

- Designed experiments (50%)
- Performed experiments (70%)
- Analyzed data (50%)
- Statistical analysis (100%)
- Manuscript writing (20%)

Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is typically caused by mutations in genes of the extrinsic FAS mediated apoptotic pathway, but for about 30% of ALPS-like patients the genetic diagnosis is lacking. We analyzed 30 children with ALPS-like disease of unknown cause and identified two dominant gain-of-function mutations of the Signal Transducer And Activator Of Transcription 3 (STAT3, p.R278H, p.M394T) leading to increased transcriptional activity. Hyperactivity of STAT3, a known repressor of FAS, was associated with decreased FAS-mediated apoptosis, mimicking ALPS caused by *FAS* mutations. Expression of BCL2 family proteins, further targets of STAT3 and regulators of the intrinsic apoptotic pathway, was disturbed. Cells with hyperactive STAT3 were consequently more resistant to intrinsic apoptotic stimuli and STAT3 inhibition alleviated this effect. Importantly, STAT3-mutant cells were more sensitive to death induced by the BCL2-inhibitor ABT-737 indicating a dependence on anti-apoptotic BCL2 proteins and potential novel therapeutic options.

1. Introduction

The Canale-Smith or autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of disturbed apoptosis [1-3]. This physiological form of cell death is of fundamental importance for the regulation of cell numbers in the immune system [4]. In human cells apoptosis is conveyed via two major pathways. In the extrinsic pathway, cell surface death receptors (FAS, TRAIL-R1/2 and TNF-R1) are activated upon binding of their cognate death ligands (FAS-L, TRAIL, TNFα) [5]. Cell death is instantly initiated by intracellular recruitment of adapter proteins and inactive procaspase-8 and -10 into a death-inducing signaling complex that mediates the activation of the caspases. A caspase cascade is triggered by cleavage mediated activation of downstream executioner caspases. Cellular proteins are proteolyzed leading to the death of so-called type I cells (e.g. activated T cells). In most cells, however, the amount of death receptor activated caspases is insufficient to induce cell death (type II cells). In these cells, the signal needs to be amplified by the intrinsic mitochondria mediated pathway through cleavage of the BCL-2 protein BID by caspase-8 or -10. This leads to the formation of active truncated BID (tBID) that translocates to the mitochondria and activates the pro-apoptotic BCL-2 family members BAX and BAK1 to mediate the release of cytochrome c from the mitochondria into the cytosol. This triggers the assembly of the apoptosome and subsequent activation of effector caspases and cell death execution. The intrinsic apoptotic pathway can also be initiated independent of the extrinsic pathway by multiple signals, including cytokines and

cellular stress. Members of the BCL2 family regulate each other through complex bimolecular interaction networks and function together as a rheostat, controlling the sensitivity of cells to apoptotic stimuli [6].

All members of the BCL2 family contain one to four conserved sequence motifs called the BCL2 homology (BH1–4) domains. Among the pro-apoptotic members of the BCL2 family, there are the so-called BH3-only proteins (including BID and BIM) that contain only the single BH3 domain [7]. The BH3 domain comprises a 9-amino acid amphipathic α-helix that binds to a hydrophobic pocket of BCL2-like anti-apoptotic proteins (including BCL2 and BCL-XL). In this manner, pro-apoptotic BH3 only proteins are kept inactive. A class of small molecule drugs (including ABT-737 [8]) mimics BH3-domain proteins and aims at disrupting this complex, thereby sensitizing cells to apoptosis.

In 70% of patients the genetic causes of ALPS are germline or somatic mutations in the genes of the extrinsic apoptotic pathway encoding the Fas receptor (*FAS*), its ligand (*FASLG*) or caspase-10 (*CASP10*) [3]. Defective death receptor signaling results in the accumulation of lymphocytes and failure to remove auto-reactive lymphocytes. As a consequence ALPS patients characteristically show enlargement of lymph nodes, spleen and liver and increased numbers of peripheral, otherwise rare terminally differentiated, activated, double-negative T lymphocytes that do not express the surface markers CD4 and CD8, but have a functional alpha/beta T-cell receptor (so called α/β-DNT cells). Although lymphoproliferation is initially non-malignant, ALPS patients have an increased risk of developing B and T cell malignancies (in particular lymphomas) early in life caused by inappropriately surviving lymphocytes with oncogenic potential [9,10]. Autoimmune disease in ALPS patients is mainly caused by B lymphocytes and directed towards blood cells. Increased numbers of B cells producing autoreactive antibodies cause various autoimmune problems: clinically significant hemolytic anemia, thrombocytopenia with bleeding tendency, autoimmune neutropenia and risk of bacterial infection. Other organs such as skin, liver, kidney, endocrine or nervous system may be affected by autoimmunity [11].

The signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family that controls apoptosis, proliferation and cell growth by regulation of gene expression in response to extracellular cytokines, IFNγ or growth hormones [12]. Somatic gain-of-function (GOF) mutations of *STAT3* cause lymphoproliferative neoplasms, aplastic anemia, and myelodysplastic syndrome [13-15], whereas germline *STAT3* gain-of-function mutations have been reported in patients suffering from recurrent infections, multi-organ

autoimmunity, hypogammaglobulinemia and lymphoproliferation, indicating a potential overlap of phenotype with ALPS disease [16-18]. The underlying molecular basis for lymphoproliferation in *STAT3* GOF caused disease is not yet understood. Due to this lack of knowledge and the lack of clinically approved STAT3 inhibitors, targeted treatment is not yet available.

2. Material and methods

2.1 Patients, relatives and healthy controls

Patients, relatives and healthy controls were enrolled in this study after obtaining written informed consent. All experiments were approved by the Ethical Review Boards of the Hadassah Hebrew University, the Israeli Ministry of Health and the University Hospital Düsseldorf. Whole-exome sequencing of DNA derived from peripheral blood was performed on a HiSeq2000 (Illumina, San Diego, CA).

2.2 Sanger sequencing of germline and somatic mutations in classical ALPS genes

To exclude germline or somatic variations in classical ALPS associated genes as genetic cause of the disease we amplified exons including exon/intron borders of *FAS*, *FASLG* and *CASP10* by PCR and carried out Sanger sequencing as described earlier [19].

2.3 Whole-exome sequencing and bioinformatic analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA for the patient and family members using the SeqCap EZ Exome Library 2.0 kit (Roche/Nimblegen, Madison, WI). 100 bp single-read sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA) essentially as described [20]. Sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA. Sequencing data was converted using Samtools. Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor, based on the Ensemble database (v70). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

2.4 Validation of *STAT3* sequence variation

Validation of the nucleotide variations in the *STAT3* gene were performed by PCR/Sanger sequencing using genomic DNA from the patients and family members. The following

primers were used: STAT3-R278H (forward: 5' CCTTGTTCTTATTGTAGTGGTCTCC 3', reverse: 5' AAAGAGAAGATGGGCTCACG 3'), STAT3-M394T (according to [17]) (forward: 5' CTTCATCCTCCGGCTACTTG 3', reverse: 5' GAGCTCCTCCCACATACCAA 3'). DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 µM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 seconds at 98°C followed by 30 cycles of 7 seconds at 98°C, 23 seconds at 60°C, 30 seconds at 72°C and a final extension of 10 minutes at 72°C. Sanger sequencing was carried out by a core facility (BMFZ, University Duesseldorf, Germany). The nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

2.5 Isolation and cultivation of mononuclear cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells were isolated using density gradient centrifugation and cultured in medium consisting of RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% fetal calf serum (FCS) and 100 µg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach, Germany). For the first 4 days, cells were activated by addition of 7 µg/ml phytohemagglutinine (PHA, Life Technologies).

2.6 Transformation of primary B lymphocytes

Cell lines of R278H mutant STAT3 B cells and healthy controls were generated by transformation with Epstein-Barr virus (EBV) (ATCC, Wesel, Germany) as described previously [21] and cultured in RPMI1640 supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies).

2.7 Immunoblotting

Cells treated with IL6 (25 ng/ml, Miltenyi) for 24 h or left untreated were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 8-15% polyacrylamide gels, transferred to polyvinylidene fluoride membranes and detected by chemiluminescence (GE Healthcare, Freiburg, Germany). The following primary antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO), STAT3 (R&D Systems, Wiesbaden, Germany), phospho-STAT3 (Tyr705, Cell Signaling, Frankfurt am Main, Germany), BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA), BCL-XL (Cell Signaling), tubulin-HRP (Cell signaling).

2.8 Luciferase reporter assays

STAT3 was cloned into the pMC plasmid [22] and a hygromycin expression cassette was added. *STAT3* variants (R278H, K392R, M394T) were generated by site-directed mutagenesis employing the Q5 site-directed mutagenesis kit as recommended by the manufacturer (NEB). The nucleotide sequence was confirmed by Sanger sequencing on an ABI 3130 Genetic Analyzer (Applied Biosystem, Carlsbad, CA). Stable human embryonic kidney (HEK) 293 cell clones expressing *STAT3* wildtype or mutant constructs, respectively, were generated by transfection using Lipofectamin LTX (Thermo Fisher Scientific, Waltham, MA) and selected with 200 µg/ml hygromycin. HEK293 cell clones were then transfected with a dual-luciferase reporter system (Cignal STAT3 Reporter, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Multiple repeats of a STAT3 binding site and basic promoter elements drive the expression of firefly luciferase. Renilla luciferase under the control of a CMV promoter serves as a control of transfection efficiency. Luciferase activity was determined in untreated cell and cells treated with either 50 µM S3I-201 (50 µM) or IL6 (10 ng/ml) after 24 h using the Dual-Glo® Luciferase Assay (Promega, Mannheim, Germany) and a Spark 10M plate reader (Tecan Group, Maennedorf, Switzerland). Dual-luciferase results were calculated for each transfectant. The change in STAT3 activity was determined by comparing the normalized luciferase activities of the reporter in cells stably expressing mutant STAT3 compared to wildtype STAT3. Assays were performed in triplicates and repeated three times. One-way-ANOVA and Fisher LSD post-hoc significance were used for calculation of statistical significance.

2.9 Real-time PCR and gene expression arrays

Primary or EBV transformed cells derived from the patients or healthy controls were cultivated in the absence of serum for 16 h. Subsequently, cells were incubated in RPMI1640 supplemented with 2% FCS with or without IL21 (100 ng/ml), IL6 (25 ng/ml), S3I-201 (50 µM) or solvens (DMSO) for 24 h. Total RNA was then isolated using the RNeasy kit (Qiagen). cDNA was synthesized employing the QuantiTect Reverse Transcription kit. Real-time PCR was performed in triplicates and repeated at least two times using QuantiTect primers for *GAPDH, ACTIN, PPIA, SOCS3, BCL2, BCL-XL, BAX, BAK, FAS, FASLG* and the QuantiTect SYBR Green RT-PCR kit according to the manufacturer's recommendations (all from Qiagen). *GAPDH*, *PPIA* and *ACTIN* expression were used as an internal standard. Further gene expression analyses were performed using the RT^2

Profiler™ PCR Array Human Apoptosis according to the manufacturer's recommendations (Qiagen).

2.10 Immunophenotyping and ELISA

Immunophenotyping was carried out during routine clinical workout. In addition ALPS specific criteria were analyzed according to standard protocols employing the following antibodies: CD3 (clone UCHT1, BD Biosciences, Heidelberg, Germany), TCR αβ (clone T10B9.1A-31, BD Biosciences), CD4 (clone VIT4, Miltenyi), CD8 (clone BW135/80, Miltenyi), B220, HLA-DR, CD27, CD19, CD25 (all from BD Biosciences) and CD45R (Beckman Coulter, Krefeld, Germany). Cell surface expression of FAS and FASL was measured using anti-CD95 (clone DX2, BD Biosciences) and anti-CD178/FasL (clone NOK-1, Miltenyi) antibodies. FASL plasma levels were measured by ELISA (R&D-Systems) employing an Infinite M200 microplate reader equipped with Magellan software (Tecan).

2.11 Measurement of apoptosis

Activated primary T cells from the patients and healthy controls or EBV transformed B cells derived from the patient and healthy controls were plated in 96 well plates at a density of 10^6 cells/ml and treated with either recombinant SuperFasLigand (100 ng/ml, Enzo Life Sciences, Lörrach, Germany), 0.5 µM staurosporine (LC Laboratories, Woburn, MA), 100 ng/ml interleukin-21 (IL21) (Miltenyi), 1 nM-10 µM ABT-737[8] (Selleckchem, Houston, TX), 10-50 µM S3I-201, mock-treated with solvens (DMSO) or left untreated. After 24 h cells were stained with Annexin V-FITC (BD Biosciences) and propidium iodide (PI, Sigma-Aldrich, Deisenhofen, Germany) and apoptosis was measured employing a FACSCalibur according to the manufacturer's suggestions (BD Biosciences) and analyzed using FlowJo software (v10, FlowJo LLC, Ashland, OR).

3. Results

3.1 Clinical characteristics of two unrelated individuals within a cohort of 30 patients with ALPS-like disease of unknown genetic cause

Patient 1 was born to consanguineous parents and suffered from early-onset hemolytic anemia, generalized lymphadenopathy and splenomegaly with subsequent development of autoimmune hypothyroidism and panhypogammaglobulinemia (Figure 1A, and Table 1). Lymph node biopsy showed hyperplasia without evidence of infection or malignancy. Repeated immunophenotyping (Table 2) revealed normal absolute numbers of T, B, and natural killer cells, but increased double negative T cells (6-20%, reference range <2.5%)

and low regulatory T cells (0.4%, reference range 1-2%) in peripheral blood. She presented with increased numbers of naïve B cells (80% IgD+CD27-, reference range 39.5-76.3%) and B cells expressing only low levels of the CD21 co-receptor (21%, reference range 1.3-7.3%). Increased immunoglobulin M (IgM) serum levels with markedly reduced levels of IgG and IgA were observed, indicating Ig isotype class switch impairment. Steroid treatment caused improvement of the hemolytic anemia, but the patient continued to suffer from progressive lymphadenopathy and splenomegaly with episodes of immune-mediated thrombocytopenia. Short stature, recurrent Herpes zoster infections, glucose intolerance and elevated intraocular pressure were regarded as complications of steroid treatment. Mycophenolate mofetil was used to decrease the steroid dose resulting in the resolution of hemolysis and thrombocytopenia.

Patient 2 was born to non-consanguineous parents and presented with non-infectious, non-malignant lymphadenopathy, splenomegaly, thrombocytopenia, neutropenia, mild anemia and early-onset insulin dependent diabetes mellitus (Figure 1B and Table 1). Double negative T cells were not elevated and immunoglobulin levels were normal (Table 2).

The presumptive diagnosis of ALPS was established for both patients.

3.2 Identification of two *de novo* heterozygous *STAT3* germline mutations

Because neither germline nor somatic mutations in classical ALPS associated genes (*FAS, FASLG, CASP10*) were detected, whole exome sequencing was carried out to clarify the genetic cause of disease. A novel heterozygous germline mutation of *STAT3* (c.833G>A, p.R278H) was gained *de novo* in patient 1 (Supplementary Table 1) and confirmed by Sanger sequencing (Figure 1C). A Combined Annotation-Dependent Depletion (CADD)-score of 23.9 indicated a deleterious mutation [24] with a high likelihood of being pathogenic (Table 1). A somatic R278H mutation was recently detected in a diffuse large B cell lymphoma [23] and an R278C mutation in an endometrium derived carcinoma (COSMIC: Catalogue of Somatic Mutations in Cancer - cancer.sanger.ac.uk/cosmic database). The affected coiled-coil domain is associated with nuclear shuttling of STAT3 and binding of cytokine receptors [24]. In this domain, one other germline gain-of-function mutation (R152W) was reported previously [18]. The patient harboring the R152W mutation presented with ALPS-like symptoms: early-onset lymphadenopathy, hepatosplenomegaly, autoimmune hemolytic anemia and thrombocytopenia, and increased DNT cells (2.7-3.5%). In addition, insulin dependent diabetes mellitus and recurrent Herpes zoster infections were observed.

In case of patient 2 whole exome sequencing revealed a previously reported *de novo* *STAT3* mutation that affected the DNA binding domain (c.1181T>C, p.M394T, Figure 1D, Supplementary Table 2) [17]. The M394T mutation of STAT3 was previously reported in one patient who presented with mild immune mediated thrombocytopenia, hypogammaglobulinemia, and disseminated mycobacterial disease in late adolescence. No autoimmunity or general lymphoproliferation, but increased numbers of DNT cells (5%) were observed [17]. In comparison, patient 2 presented in this study had a more severe phenotype, but did not suffer from mycobacterial disease or eczema.

3.3 The identified mutations confer elevated constitutive and inducible activity to the transcription factor STAT3

To analyze the impact of the identified mutations on the transcription factor function of STAT3, human embryonic kidney (HEK) 293 cells stably expressing STAT3 wildtype, the STAT3 variants R278H and M394T and STAT3 K392R as a positive control were generated and dual-luciferase reporter assays were carried out using promoter elements with multiple repeats of a STAT3 binding site that drive the expression of firefly luciferase. Luciferase activity was determined in untreated cells and cells treated with a cell permeable, small molecule inhibitor of STAT3 targeting the SH2 domain: S3I-201 (2-hydroxy-4-[[[[(4-methylphenyl)sulfonyl]oxy]acetyl]amino]-benzoic acid, NSC 74859). It was recently demonstrated that the inhibitor prevents phosphorylation, activation, dimerization, and DNA-binding of STAT3.[25] Compared to wildtype STAT3, both patient derived mutants analyzed were transcriptionally increased active. M394T mutant STAT3 has previously been shown to increase luciferase expression placed under a STAT3-specific promoter compared to wildtype controls [17]. Activity of both mutant and wildtype STAT3 could be diminished by application of the STAT3 inhibitor (Figure 1G). After stimulation with IL6, the transcriptional activity of both wildtype and mutant STAT3 was highly increased, but mutant STAT3 still largely exceeded the activity of wildtype STAT3 (Figure 1H). Taken together, both STAT3 mutations detected in ALPS like patients seem to confer constitutive activity to the transcription factor STAT3.

Phosphorylation at tyrosine residue 705 is required for dimerization and activation of STAT3 [15]. To determine whether the identified mutations have an impact on protein function we analyzed the phosphorylation state of STAT3 in patient cells. B cells of patient 1 and healthy controls were immortalized with Epstein Barr virus and lysates of these cells were used for western blot analyses.

pSTAT3 (Tyr705) levels were elevated in transformed R278H mutant cells compared to transformed control cells indicating that the R278H mutant is constitutively more active (Figure 2A). To our knowledge we demonstrate for the first time constitutive phosphorylation of STAT3 in primary cells of a patient: increased phosphorylation of STAT3 was observed under basal untreated conditions in lysates prepared from primary T cells of patient 2 (harboring the M394T mutation) compared to primary healthy control T cells. Treatment with interleukin-6 (IL6) led to increased STAT3 phosphorylation in healthy control cells indicative of activation, but did not further elevate the pSTAT3 (Tyr705) signal in cells derived from patient 2. Therefore, both detected *de novo* STAT3 mutations seem to confer constitutive phosphorylation to the transcription factor STAT3.

To test this finding, we examined the expression of known STAT3 target genes. A main target of STAT3 is the suppressor of cytokine signaling 3 (SOCS3). We first isolated RNA from transformed B cells of patient 1 both under basal, untreated conditions and after activating STAT3 signaling by treatment with interleukin-21 (IL21) and carried out real-time PCR analyses. The results showed that *SOCS3* expression was highly increased in R278H mutant cells under basal and even more under stimulated conditions and could be decreased by addition of the STAT3 inhibitor S3I-201 (Figure 2B, C). Similarly, *SOCS3* expression was upregulated in M394T mutant primary T cells derived from patient 2 compared to primary T cells from healthy controls under basal conditions and more enhanced after activation of STAT3 signaling by IL6 treatment (Figure 2D). Germline gain-of-function mutations have been identified in all functional domains of STAT3 in humans. The majority of these mutations are situated in the DNA binding region and probably impact on the DNA-protein interaction (Figure 2E) [26]. Other functions including stabilization of phosphorylation, dimerization, interaction with other proteins or nuclear shuttling may also be affected. The molecular basis underlying the gain of function of these mutant STAT3 proteins is currently unknown.

3.4 Patient derived cells expressing hyperactive STAT3 are resistant against diverse apoptotic stimuli

Because clinical symptoms observed in the analyzed patients can be caused by defective lymphocyte cell death mediated by the FAS signaling pathway, we analyzed the expression of the FAS ligand and FAS receptor and tested the efficiency of FAS ligand mediated apoptosis in cells derived from the patients (Figure 3A, B).

In patient 2 we detected a decrease of *FASLG* expression on RNA, but not on protein level employing flow cytometric detection of the FAS ligand protein at the cell surface (data not

shown). Serum levels of soluble FASL were not significantly altered (Table 2). Using gene expression arrays, real-time PCR and flow cytometric analyses we detected a decrease in RNA and protein expression of the FAS receptor in M394T mutant primary T cells (Figure 3C and Supplementary Figure 1). These findings indicated a possible deficiency of FAS receptor mediated cell death. To test this hypothesis in primary cells derived from both patients we used recombinant FASL to induce apoptosis *in vitro*. Primary T cells from the patients were indeed significantly less sensitive to recombinant FASL in vitro compared to controls confirming a deficiency of the FAS mediated apoptotic pathway (Figure 3B). Interestingly, treatment of M394T mutant cells with STAT3 activating IL6 further protected cells from FasL induced apoptosis (Figure 3D). To check whether the apoptosis defect was indeed associated with activated STAT3, we repeated the apoptosis tests using R278H mutant transformed B cells and pre-treated the cells with the specific, cell permeable STAT3 inhibitor S3I-201[25] prior to addition of recombinant FASL. Indeed, the inhibition of STAT3 restored the apoptotic response of R278H mutant cells to FASL (Figure 3E). This effect was not observed when primary M394T cells were similarly treated (Figure 3F). This may be due to a lower effect of the S3I-201 inhibitor on M394T mutant STAT3 as indicated in reporter assays (Figure 1G) or the higher sensitivity of cells expressing this mutant to FASL mediated apoptosis (Figure 3B).

Because FAS mediated apoptosis is amplified in various cell types by the intrinsic mitochondrial apoptotic pathway, we also tested the functionality of this pathway in the patient cells using specific stimuli. Interestingly, transformed R278H mutant B cells were also clearly more resistant to intrinsic apoptotic stimuli like staurosporine or IL21 than comparable controls (Figure 4A and Supplementary Figure 2). This finding indicates that a combined defect of both the extrinsic and the intrinsic apoptotic pathway may cause the resistant phenotype of STAT3 mutant patient cells.

3.5 Constitutive activation of the transcription factor STAT3 tips the balance of pro- and anti-apoptotic proteins of the BCL2 family to an apoptosis resistant state

Intrinsic apoptosis is tightly regulated by BCL2 proteins. To assess whether constitutively active STAT3 is associated with a disturbance of the balance of BCL2 proteins in patient cells, we carried out gene expression studies employing real-time PCR and expression arrays. In transformed R278H mutant B cells increased expression of resistance mediating anti-apoptotic BCL2 family members *BCL2* and *BCL-XL* (synonymously *BCL2L1*, Figure 4B) was detected and confirmed on protein level employing immunoblot analyses (Figure 4C). Expression of BCL2 family members that promote apoptosis, including *BAX* and *BAK1* was

downregulated in R278H mutant cells compared to controls (Figure 4B). Similarly, in primary M394T mutant T cells *BCL2* RNA levels were upregulated and *BAX* and *BAK1* downregulated (Figure 4B). However, *BCL-XL* was not significantly differently expressed in M394T cells, indicating that different STAT3 mutations may elicit different disturbances of BCL2 protein family expression. Taken together our results indicate that the balance of pro- and anti-apoptotic BCL2 family proteins is tipped in favor of anti-apoptotic proteins rendering the patient derived cells more resistant to diverse apoptotic stimuli.

To test whether the alteration of the expression pattern is associated with STAT3 activation, we employed the STAT3 inhibitor S3I-201. IL-21 mediated apoptosis was associated with a downregulation of anti-apoptotic and upregulation of pro-apoptotic BCL2 family members in transformed B cells derived from healthy controls that was less pronounced in STAT3 mutant cells (Figure 4D). Similarly, expression of *BCL2* was increased in M394T mutant cells upon stimulation with IL6 (Figure 4E). Administration of the STAT3 inhibitor re-adjusted the expression of most BCL2 family proteins in transformed R278H cells to levels comparable to normal cells (Figure 4F). Pre-treatment with S3I-201 restored the apoptotic response of these cells to IL21 (Figure 4G).

3.6 Cell death of lymphocytes expressing hyperactive STAT3 can be restored by the BCL2-inhibitor ABT-737

Our data suggested that activating *STAT3* mutations induce resistance to apoptosis by tipping the balance of BCL2 family members in favor of anti-apoptotic proteins. BH3 mimetic drugs such as the small molecule ABT-737 target the BH3-protein binding cleft of BCL2-like anti-apoptotic proteins and release sequestered BH3 proteins to induce apoptosis [8]. Via this mechanism ABT-737 efficiently kills cells that are dependent on *BCL2* expression for survival. Both R278H and M394T mutant cells overexpressed *BCL2*. We therefore tested whether the patient cells with constitutively active STAT3 respond to this drug. Both transformed R278H mutant cells (Figure 5A) as well as primary M394T mutant T cells (Figure 5B) were significantly more sensitive to ABT-737 compared to healthy control cells. In primary cells the effective dose was in the low nanomolar range and showed a considerably wide therapeutic window.

4. Discussion

Patients with hyperactive or constitutively active STAT3 share common clinical characteristics with ALPS patients, including lymphoproliferation and autoimmune cytopenias. ALPS patients frequently have increased IL10 plasma levels probably leading

to hyperactive STAT3 signaling.[27] ALPS and STAT3 gain-of-function both predispose to lymphoid malignancies [9,10,14]. Interestingly, diffuse large B cell lymphoma may present with persistent STAT3 activation due either to elevated IL10 levels, STAT3 gain-of-function mutations or upstream events [28-30].

The mechanistic cause of lymphoproliferation and perturbed apoptosis induction in STAT3 hyperactive syndrome has, however, not yet been characterized. ALPS patients consistently present with elevated numbers of DNT cells, but DNT cells may as well be increased in patients affected by other immune dysregulation syndromes [31] and in STAT3 hyperactive cases

(5 of 13 [18], 1 of 3 [17], and 1 of 2 cases in the present study). We therefore assumed that hyperactive STAT3 could block lymphocyte apoptosis by deregulating apoptotic signaling pathways involved in the pathogenesis of ALPS or ALPS-like disease. An increase in lymphoproliferation caused by STAT3 hyperactivity was not observed in our study.

Deregulation of *FAS* was a likely cause for the ALPS-like symptoms of the patients in our study. Indeed, we could demonstrate that *FAS* expression was decreased in patient 2 and FAS mediated apoptosis was deficient in primary T cells derived from both analyzed patients. This effect was probably closely linked to STAT3 activity in the cells of our patients, because inhibition of STAT3 in R278H cells, using the small molecule STAT3 inhibitor S3I-201, led to reconstitution of FAS mediated apoptosis.

It has previously been demonstrated that STAT3 can repress *FAS* transcription [32]. The *FAS* gene promoter contains binding sites for STAT3 (-460 to -240 bp) and direct binding of STAT3 in complex with c-Jun, has been confirmed using EMSA and ChIP assays. It is probable, that distinct mutations may affect STAT3 function in different ways. In this line Milner et al. [18] observed that mutations in the DNA binding domain caused constitutive activation of STAT3, whereas a mutation (p.K658N) in the dimerization domain only conferred hypersensitivity to interleukins. Depending on the effect of the respective STAT3 mutation on e.g. target recognition or interaction with co-factors, distinct mutations of *STAT3* may achieve different levels of suppression of *FAS* expression and FAS mediated apoptosis. In their cohort of 15 patients with hyperactive STAT3 and without common ALPS mutations, Milner et al. [18] tested susceptibility to FAS mediated apoptosis in activated primary cells expressing five (of nine) different *STAT3* mutations (p.T663I, p.R152W, p.V353F, p.N420K, p.A703T). Strikingly, p.A703T mutant cells showed no significant cell death induction at all after stimulation with an agonistic FAS antibody (CH11) compared to controls. A similar effect was reportedly not observed for the other

mutations, but the data was not included. Our data showed a partial defect in FAS mediated apoptosis, therefore the difference between our studies may be due to different cut offs. Treatment of the patients at the time of sample drawing might also have adversely affected the assay. Nevertheless, taken together, these findings strongly indicated a possible impact of disturbed FAS receptor signaling on the clinical symptom of lymphoproliferation in at least some patients with STAT3 hyperactivity, but also suggested that other additional mechanisms may play a role.

Our results provided evidence for a dysregulated BCL2 pathway as an important mechanism for lymphoproliferation and autoimmunity in patients with *STAT3* gain-of-function mutations. The anti-apoptotic state of the cells seemed to be closely correlated with an overexpression of anti-apoptotic BCL2 proteins. Interestingly, the main anti-apoptotic BCL2 proteins (BCL-2, BCL-XL and MCL-1) are all direct targets and positively regulated by the STAT3 transcription factor [33-36]. STAT3 is known to maintain T cell homeostasis by regulating the expression of pro-apoptotic and anti-apoptotic factors such as BCL2, BCL-XL, BAX, BAK1 and BIM. Recently, it could be demonstrated that the number of thymocytes and T cells in the spleen and lymph nodes of STAT3-deficient mice was significantly decreased due to a higher susceptibility to apoptosis [37]. Interestingly, expression of *BCL2* and *BCL-XL* was significantly reduced in STAT3-deficient thymocytes and T cells. Moreover, it could be shown that STAT3 enhances survival of activated T cells by upregulating the expression of *BCL2* while downregulating the expression of *FASL* and *BAD* [38]. STAT3 seems to function as an anti-apoptotic factor, especially in numerous malignancies, where STAT3 is constitutively active/phosphorylated [39,40].

It was recently demonstrated that the BCL2 pathway was deregulated in DNT cells of 12 ALPS patients and that the release of overexpressed BIM by the BCL2 inhibitor ABT-737 rendered the cells susceptible to apoptosis [27]. Similarly, in our study a deregulation of BCL2 family proteins sensitized the cells to this inhibitor. Our data therefore implicate BH3 mimetics as potential therapeutics for patients with *STAT3* gain-of-function mutations. ABT-737 was effective and well tolerated in mice models of autoimmune disease [41]. Side effects were restricted to dose dependent lymphopenia and thrombocytopenia. The BCL2 specific, synthetic derivative ABT-199, currently in clinical development (phase II and III trials), is an alternative drug devoid of toxicity against platelets and targeted delivery could further improve treatment specificity. It would further be interesting to see whether BH3 mimetics would also address the tumor predisposition in STAT3 gain-of-function mutated patients.

5. Acknowledgements

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6. Tables

Table 1: Clinical characteristics of the patients. Abbreviations: ANA, antinuclear antibodies; CADD score, Combined Annotation-Dependent Depletion score; CRP, C-reactive protein; DNT cells, double negative T cells ($CD4^-CD8^-TCR\alpha/\beta^+$ T cells); ESR, erythrocyte sedimentation rate; RF, rheumatoid factor

	Patient 1	Patient 2
STAT3 mutation	p.R278H *de novo*	p.M394T *de novo*
CADD score	23.9	25.4
Sex	Female	Female
Age at presentation (years)	3	2
Hematology	Chronic, generalized, non-infectious, non-malignant lymphadenopathy and splenomegaly, thrombocytopenia, intermittent leukopenia, anemia, hypogammaglobulinemia, DNT cells chronically elevated (6-20%)	Chronic, generalized, non-infectious, non-malignant lymphadenopathy and splenomegaly, thrombocytopenia and neutropenia. Mild anemia due to hemoglobin C trait.
Autoimmunity	Coombs positive hemolytic anemia, immune-mediated thrombocytopenia, auto-antibodies (ANA, anti-cardiolipin, thyroidites), RF, CRP and ESR chronically elevated	Insulin dependent diabetes, immune-mediated thrombocytopenia and neutropenia
Infection	Recurrent herpes zoster infection	No infections
Other clinical findings	Short stature, glucose intolerance, elevated intraocular pressure	Upper airway obstruction due to adenoid-tonsillar hypertrophy

Table 2: Laboratory characteristics of the patients. Variations from reference values are indicated in bold. Abbreviations: ANC, absolute neutrophil count; CBC, complete blood cells; CRP, C-reactive protein; HgB, hemoglobin; ND, not done; Ref. values, reference values; sFASL, soluble Fas ligand in plasma; WBC, white blood cells

	Patient 1	**Patient 2**	**Ref. Values**
STAT3 mutation	p.R278H	p.M394T	
Total Immunoglobulins:			
IgM (mg/dL)	**1460-2230**	**31**-208	40-147
IgG (mg/dL)	**<150-217**	**572**-1470	680-1530
IgA (mg/dL)	**<22.8**	**27**-195	66-299
IgE (Iu/mL)	48	ND	<100
B cells (of gated lymphocytes)			
CD19+ (B cells) (%)	**3**-6	24	6-25
Kappa/Lambda ratio	**1.0**-1.6	1.2	1.2-2.9
IgD+/CD27- (naïve B cells) (% of CD19+)	**80.9**	ND	39.5-76.3
CD27+ (intermediate and memory B cells) (% of CD19+)	8.9	ND	7-20
CD21 low	**21.7**	ND	1.3-7.3
T cells (of gated lymphocytes)			
CD3+ (T cells) (%)	81-**93**	72%	55-84
CD4+/CD3+ (T Helper) (%)	**22**-50.0	45%	31-60
CD8+/CD3+ (T Suppressor) (%)	35-**67**	**55%**	13-41
Ratio CD4/CD8	**0.5**-1.4	**0.8**	1-3.6
DNT cells (% of CD3+/TCRα/β+)	**6-20**	<2.5%	<2.5%
B220+ (% of DNT)	10.3	ND	8-40%
CD25+ (% of CD3+)	9.1	ND	<18%
HLA-DR+ (activated T cells, % of CD3+)	**33-38.4**	ND	<10
CD4+/CD25+ (regulatory T cells, % of CD3)	**<1%**	ND	1-2
CD56+ (Natural Killer cells) (%)	**1**-12	ND	5-27
CD25+/HLA-DR+ ratio	**0.23**	ND	2
Other parameters			
CRP	<0.32-**10.6**	**<5-8.7**	<1
WBC from CBC (10^3/μL)	**3.7**-5.2	**2.2**-4.3 (5-**13**)	4.3-10
Platelets (10^3/μL)	**57**-221	**4-55**	150-400
ANC (10^3/μL)	**0.9-6**	**0.5**-4	2-7
HgB (gr/dL)	**5.5**-15.5	**9.4**-12	10.5-15.0
sFASL (pg/ml plasma)	225	ND	<200

7. Figures

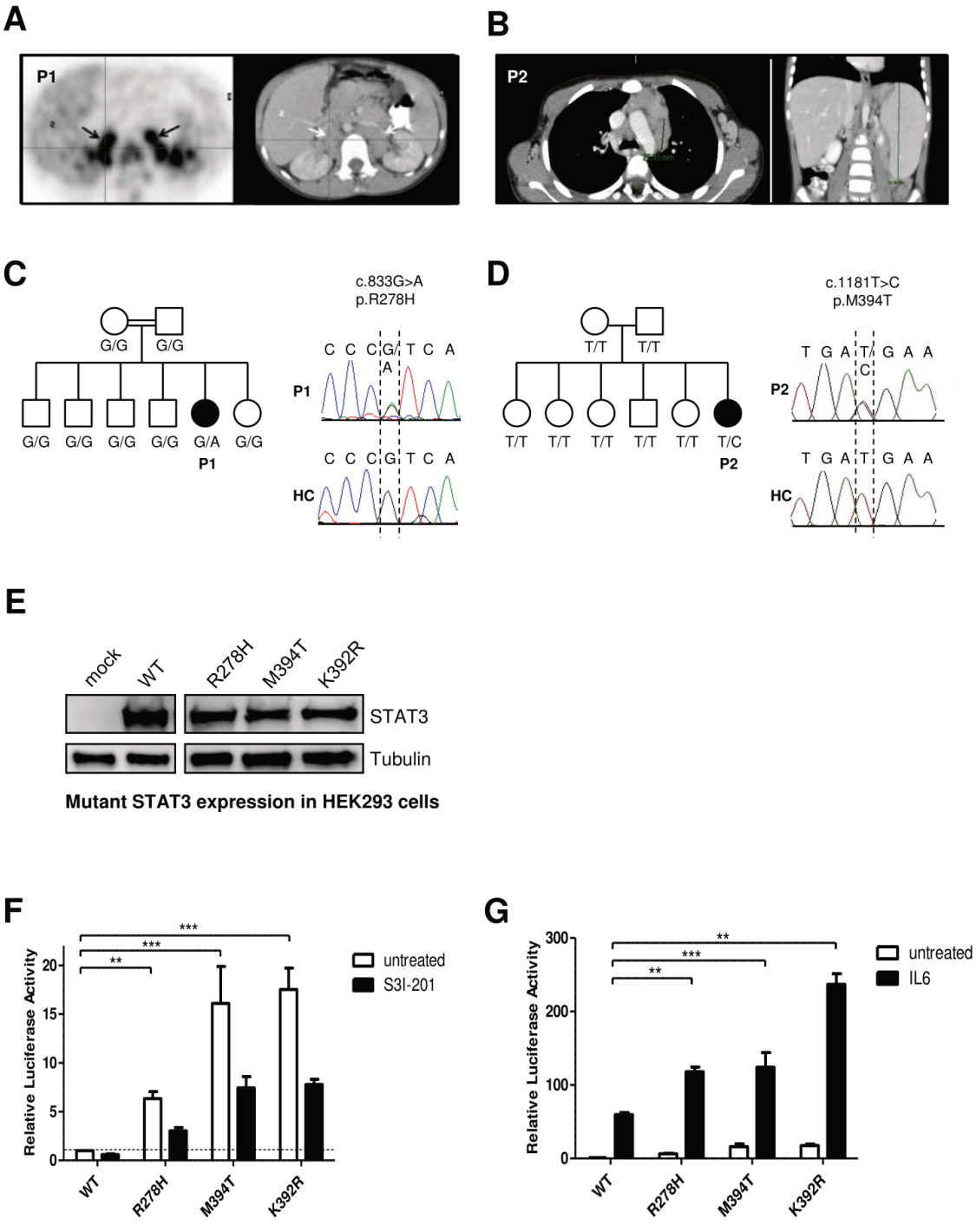

Figure 1: Two *de novo STAT3* gain of function mutations were detected in ALPS-like patients. (A, B) Computed tomography of patient 1 (P1, A) and patient 2 (P2, B) demonstrates lymphadenopathy and splenomegaly. Coronal reformatted image of the abdomen reveals significant enlarged retroperitoneal lymph nodes of P1 (arrows). Lymphadenopathy and splenomegaly of P2 are indicated by green bars. **(C, D)** Family pedigrees of P1 (C), and P2 (D). P1 descended from consanguineous, P2 from non-consanguineous parents. P1 and P2 are the only carriers of the *STAT3* mutations in their families (genotype indicated for each family member) and the only diseased family members (diseased state indicated by filled circle). Capillary

sequencing of *STAT3* confirmed *de novo* mutations in patient P1 (C) and P2 (D). **(E)** The identified *STAT3* mutations, a known detrimental *STAT3* mutation (K392R) as well as *STAT3* wildtype (WT) were cloned and expressed in HEK293 cells that lack intrinsic *STAT3* expression. Expression was confirmed by western blot employing a STAT3 specific antibody and tubulin as a loading control. Mock transfected cells show absent baseline expression of STAT3 **(F)** STAT3 reporter assays in HEK293 cells stably expressing STAT3 wildtype and variants demonstrate hyperactivity of the patient derived mutations compared to wildtype STAT3. The activity of both mutant and wildtype STAT3 could be reduced by addition of the STAT3 inhibitor S3I-201. Mean values and standard deviations are shown (**p<0.01, ***p<0.001). **(G)** STAT3 reporter assays in HEK293 cells stably expressing STAT3 variants performed as in (F) demonstrate that the activity of wildtype and mutant STAT3 is highly increased in response to IL6 (10 ng/ml, 24 h) (**p<0.01, ***p<0.001).

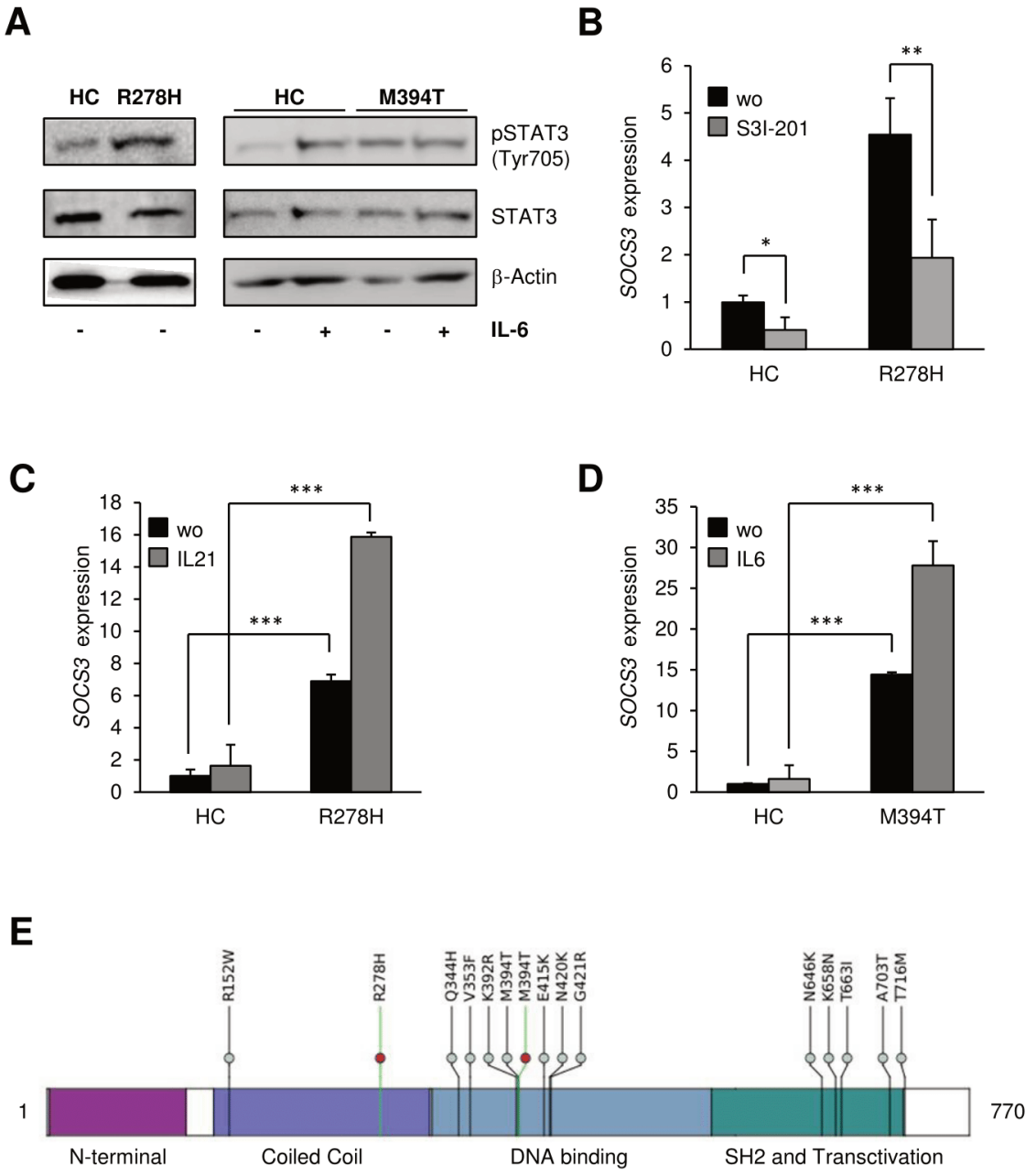

Figure 2: Increased STAT3 phosphorylation and expression of the downstream STAT3 target *SOCS3* further indicate gain of function mutations. (A) STAT3 phosphorylation at tyrosine 705 indicative of STAT3 activation is increased in cells derived from P1 and P2. Immunoblot analyses of total and phosphorylated (Tyr705) STAT3 protein employing specific antibodies is shown. β-Actin served as a loading control. Left panel: Lysates were prepared from EBV transformed B cells of a healthy control (HC) and patient P1 (harboring the R278H mutation). Right panel: Lysates were prepared from primary T cells of a healthy control and patient P2 (harboring the M394T mutation) after treatment with IL6 (25 ng/ml) for 24 h or untreated. **(B)** Expression level of the STAT3 target *SOCS3* is constitutively elevated in EBV transformed R278H B cells compared to EBV transformed healthy B cells (HC) and can be decreased by addition of the STAT3 inhibitor S3I-201 or **(C)** further increased by treatment with IL21 (100 ng/ml, 24 h). *SOCS3* mRNA expression was measured in triplicates by real-time PCR. Mean values and standard deviations are shown (*p<0.05, **p<0.01, ***p<0.001). **(D)** *SOCS3* expression is upregulated in M394T mutant primary T cells from patient P2 compared

to primary T cells from a healthy control (HC). *SOCS3* expression is further increased by treatment with IL6 (25 ng/ml, 24 h). Real-time PCR measurement was carried out as described in (B, C). **(E)** Schematic drawing of the STAT3 protein. The mutations identified in the patients (red dots) as well as other previously reported germline gain of function mutations are indicated (grey dots). The plot was generated using The Protein Painter application in the Pediatric Cancer Genome Project (http://explore.pediatriccancergenomeproject.org).

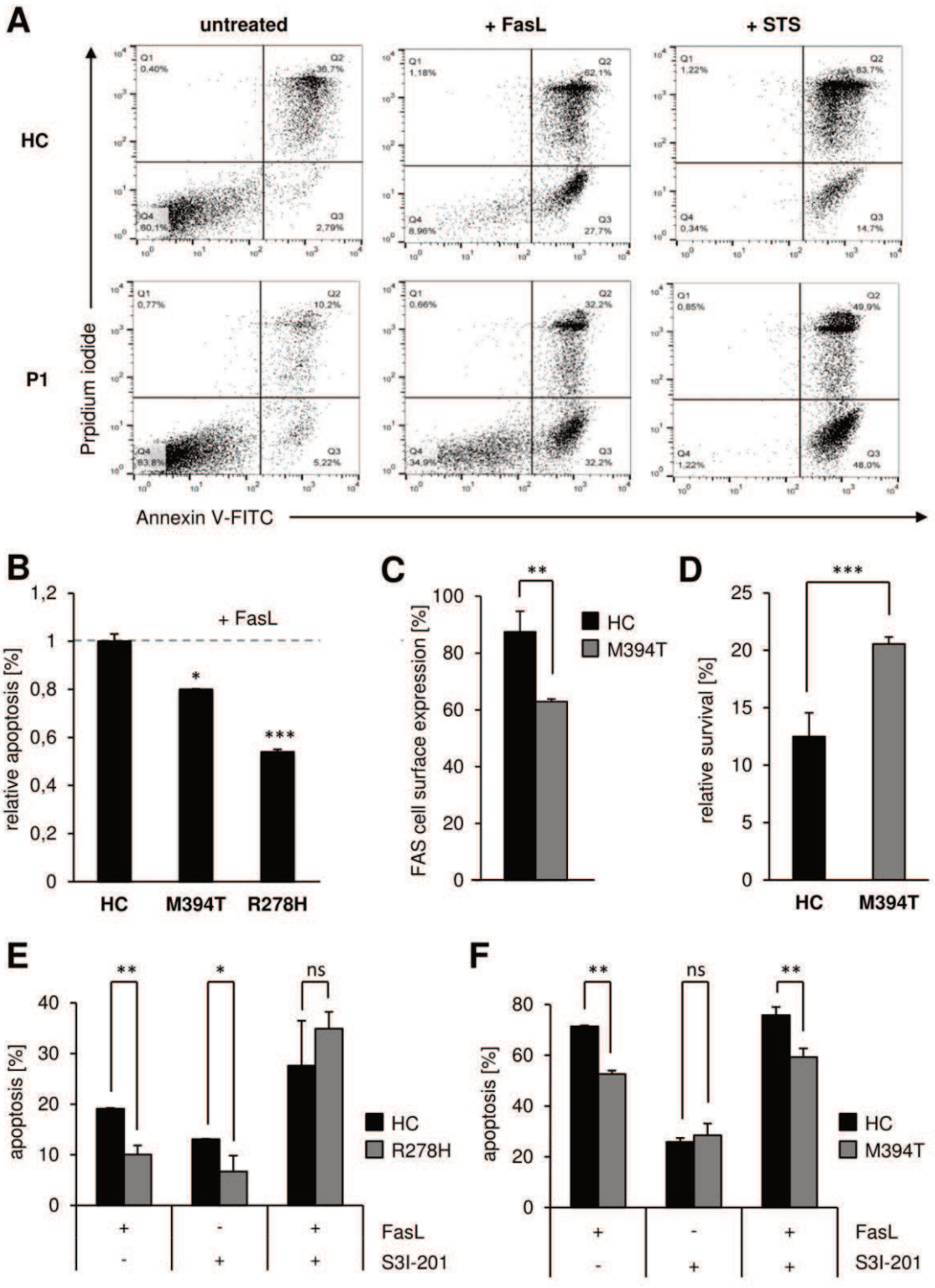

Figure 3: STAT3 mutant patient cells are less responsive to FAS ligand induced apoptosis. **(A)** Representative apoptosis measurement of primary T cells derived from patient P1 compared to a healthy control (sibling). Apoptosis was determined in untreated cells and cells treated for 24 h with either FAS ligand (FASL, 100 ng/ml) or staurosporine (STS, 0.5 µM) by flow cytometric measurement of phosphatidyl serine exposure employing Annexin V-FITC and propidium iodide. **(B)** Primary T cells derived from the patients induce apoptosis less efficiently than comparable healthy control cells. Primary T cells derived from peripheral

blood of the patient and healthy controls were activated and expanded by PHA and IL2 treatment. Apoptosis was triggered by recombinant FAS ligand (100 ng/ml for 24 h) and measured by flow cytometry using Annexin V-FITC/propidium iodide staining. Depicted is the relative difference in the apoptosis rate of treated patient to corresponding treated wildtype cells (=1). Mean values and standard deviations of a representative experiment of three, performed in duplicates, are shown (*p<0.05, ***p<0.001). **(C)** Activated primary T cells of patient P2 (M394T mutant) express decreased levels of the death receptor FAS on their cell surface. FAS expression was measured by flow cytometry employing a FAS specific antibody. Mean values and standard deviations of a representative experiment of three are shown (**p<0.01). **(D)** IL6 protects M394T mutant patient cells from FASL mediated apoptosis. Apoptosis was measured as in (B). Cells were treated with FASL in the presence and absence of IL6 (25 ng/ml). Presented is the relative survival of FASL treated M394T primary T cells in the presence versus the absence of IL6. Mean values and standard deviations of a representative experiment of two are shown (***p<0.001). **(E)** Administration of the STAT3 inhibitor S3I-201 rescues the apoptotic response of R278H cells to FASL. Transformed R278H and healthy control cells were either mock-treated with solvent or treated for 24 h with S3I-201 (50 μM). Subsequently cells were treated with recombinant FASL (100 ng/ml) for further 24 h. Cells were harvested and apoptosis analyzed as described in (A). Mean values and standard deviations of a representative experiment of three are shown (ns, not significant, *p<0.5, **p<0.01, ***p<0.001). **(F)** S3I-201 does not further increase the apoptotic response of M394T primary T cells to FASL compared to healthy control cells. Representative experiment carried out as described in (E).

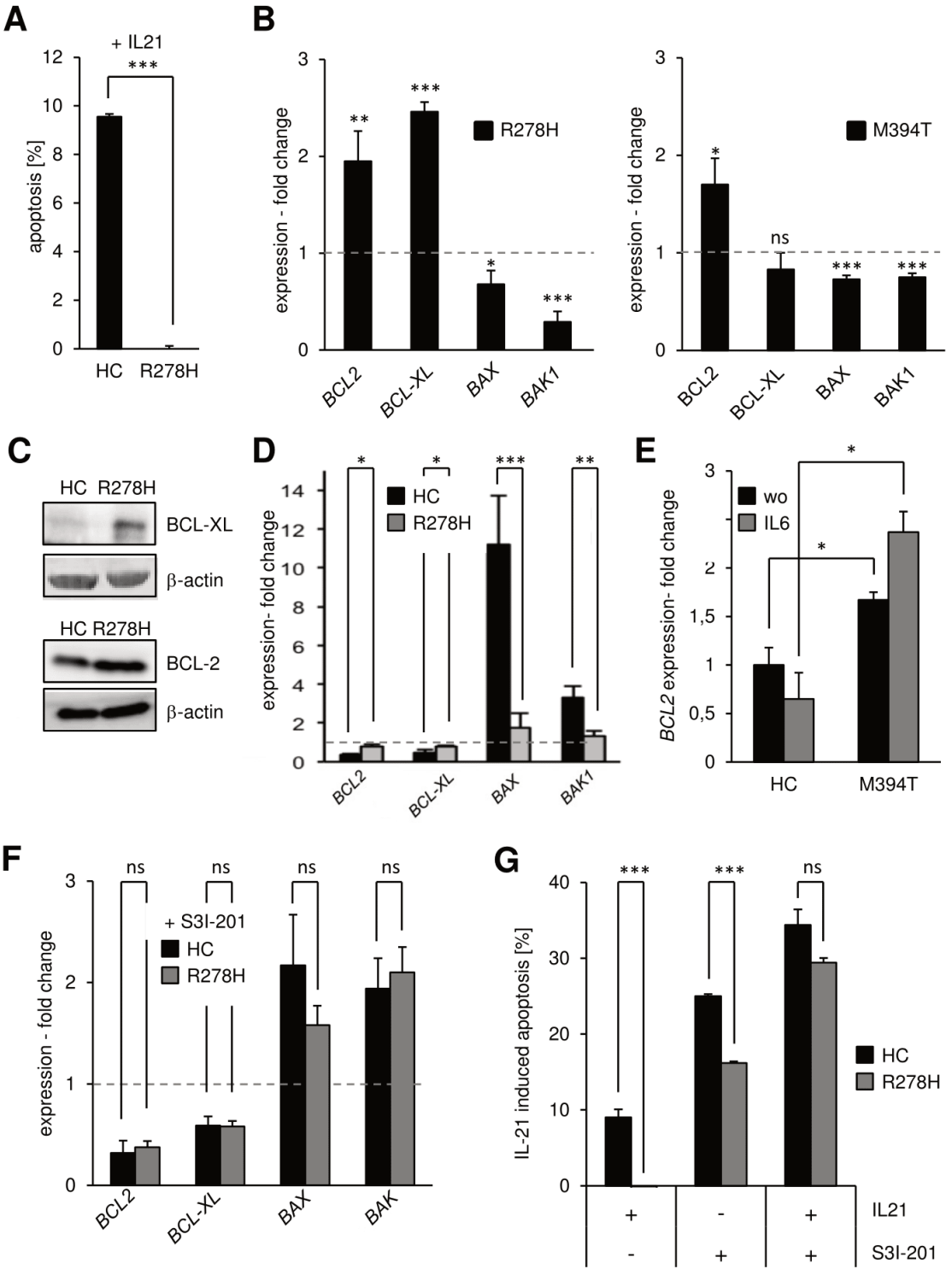

Figure 4: *STAT3* gain of function mutations protect lymphocytes from apoptosis by dysregulation of the BCL-2 pathway. (A) EBV transformed R278H B cells are less susceptible to apoptosis induced by IL21 (100 ng/ml, right panel). Apoptosis was measured after 24 h of treatment as described in Figure 3A. Mean values and standard deviations of a representative experiment of three are shown (***p<0.001). **(B)** Constitutive activation of STAT3 shifts the balance of pro- and anti-apoptotic BCL-2 proteins in favor of apoptosis resistance. Left panel: Increased expression of anti-apoptotic *BCL2, BCL-XL*, and decreased expression of pro-apoptotic *BAX* and *BAK1* is demonstrated in EBV transformed R278H B cells compared to transformed control

B cells by realtime-PCR. Experiments were carried out as described in (Figure 2B, C) and the results of the R278H mutant cells are normalized relative to the healthy control (=1) (*p<0.5, **p<0.01, ***p<0.001). Right panel: Similar measurements were carried out using M394T mutant and wildtype primary T cells. **(C)** Protein expression of anti-apoptotic BCL-2 proteins is elevated in transformed R278H cells compared to healthy control cells. Cell lysates were prepared and immunoblot analyses carried out as described in Figure 1F. Specific antibodies against BCL2, BCL-XL, and β-Actin were employed. **(D)** Impaired induction of apoptosis by IL21 in transformed R278H cells was associated with a disturbed IL21 induced downregulation of *BCL2* and *BCL-XL* and upregulation of *BAX* and *BAK1* expression. Results of a representative real-time PCR experiment carried out three times in triplicates as described in Figure 2B, C are shown. The results were normalized to the untreated results (=1) (*p<0.05, **p<0.01, ***p<0.001). **(E)** *BCL2* expression is downregulated in primary wildtype T cells in response to IL6, but further increased in primary M394T T cells. Representative real-time PCR experiment as described in (D). **(F)** Administration of the STAT3 inhibitor S3I-201 normalizes the expression of BCL2 family genes in R278H cells treated with IL21. Transformed R278H and healthy control cells were either mock-treated with solvent or treated for 24 h with S3I-201 (50 μM). Subsequently, cells were treated with IL21 (100 ng/ml) for 24 h. Cells were harvested, RNA isolated and real-time PCR analyses carried out as described in Figure 2B. Results were normalized to the results without added inhibitor. Mean values and standard deviations of a representative experiment of three are shown (ns, not significant). **(G)** Administration of the STAT3 inhibitor S3I-201 rescues the apoptotic response of R278H cells to IL21. Cells were treated as described in (F) and apoptosis was measured as described in Figure 3A. Mean values and standard deviations of a representative experiment of three are shown.

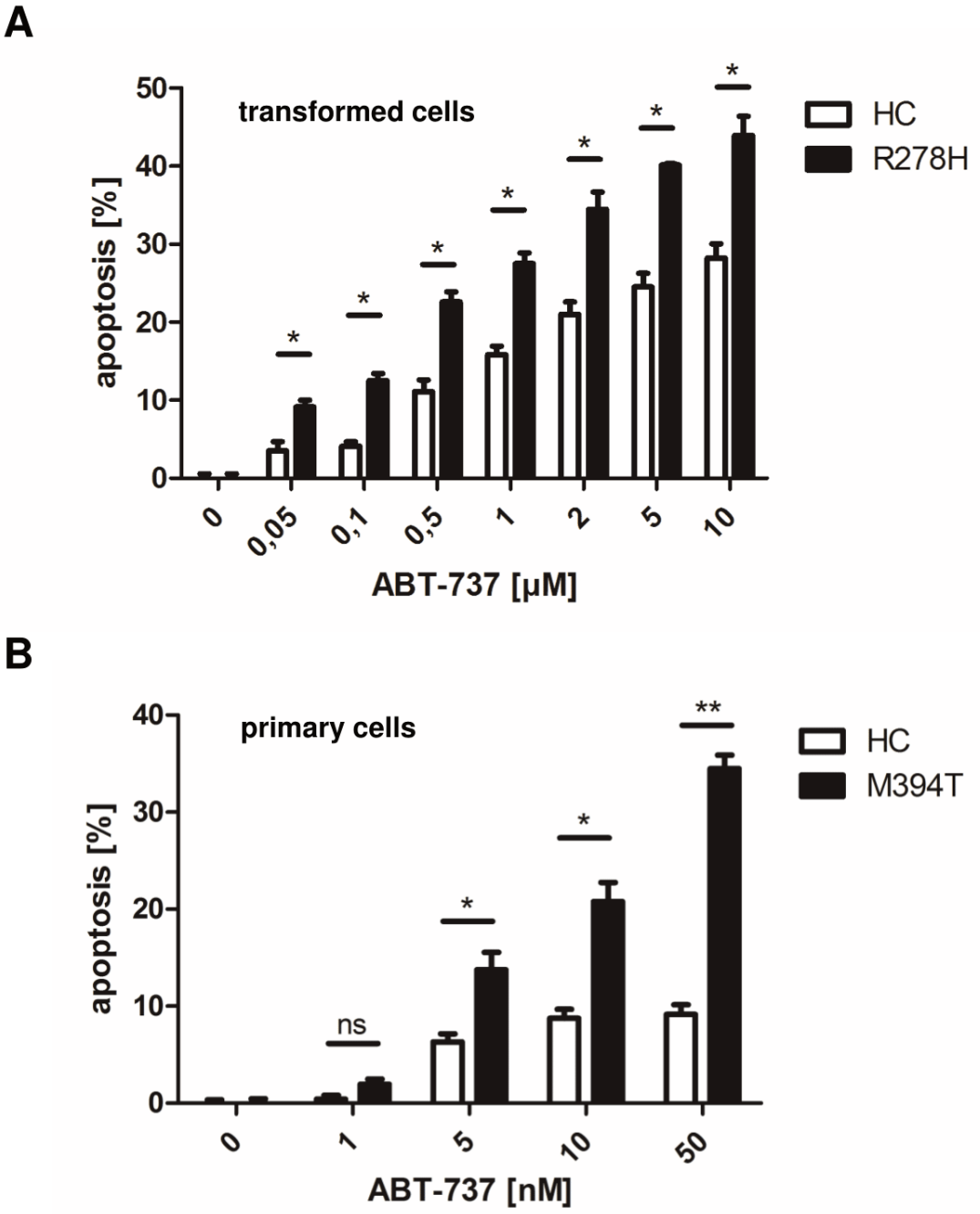

Figure 5: The cell death defect caused by activated STAT3 can be compensated by a BH3 mimetic compound. (A) Transformed R278H mutant B cells are significantly more sensitive to ABT-737 than transformed wildtype B cells. Cells were either mock-treated with solvent or treated for 24 h with the indicated concentrations of ABT-737. Subsequently, apoptosis was determined as described in Figure 3A. Mean values and standard deviations of a representative experiment of three are shown. Unpaired t-test was applied (*$p<0.05$). **(B)** Primary M394T mutated T cells are significantly more sensitive to ABT-737 than comparable healthy primary T cells in a nanomolar range. Cells were treated as described in (A) with the indicated concentrations of ABT-737. Apoptosis was determined as in Figure 3A. Mean values and standard deviations of a representative experiment of two are shown. Unpaired t-test was applied (ns, not significant, *$p<0.05$, **$p<0.01$).

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9. Supplemental Tables

Supplemental Table 1: Gene candidates identified in patient 1 by whole-exome sequencing Whole exome sequencing was carried out on DNA isolated from blood samples derived from the patient, the unaffected parents and three healthy siblings. DNA samples of further two healthy siblings were used for validation. The following filtering steps were applied: Shown are mutations that were present in the patient, but not in the parents or one of three unaffected siblings. Coverage was at least 10x and variation call quality above 30. The minor allele frequency was below 1 in 10,000 and the variation was predicted to be detrimental for protein function by the bioinformatics tools Sift or PolyPhen2. Abbreviations: Ref., reference; Alt., alteration.

Localization	Ref	Alt	Name	Genotype	Polyphen	Sift	Protein Alt.
10:93572811	G	A	*TNKS2*	1/1	probably damaging	deleterious	p.Gly91Ser
17:40486032	C	T	*STAT3*	0/1	probably damaging	tolerated	p.Arg278His

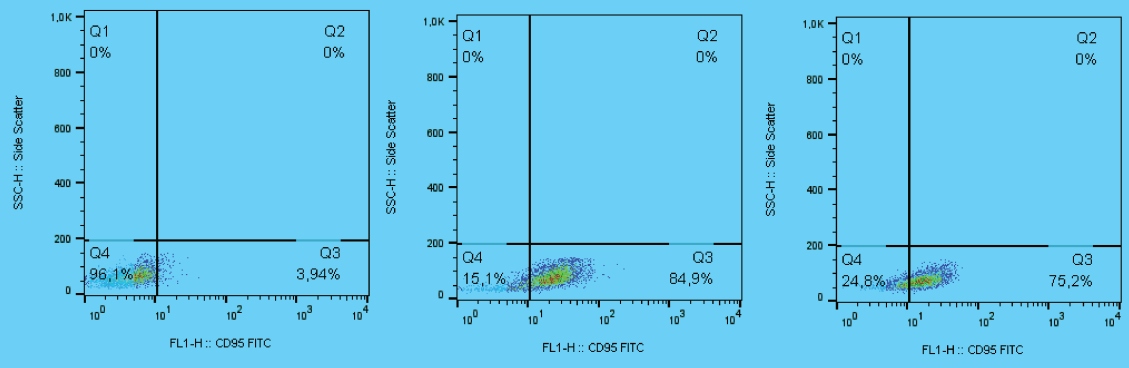
Supplemental Table 2: Gene candidates identified in patient 2 by whole-exome sequencing Whole exome sequencing was carried out on DNA isolated from blood samples derived from the patient, the unaffected mother and one healthy sibling. DNA samples of the father and further siblings were used for validation. Filter steps applied were the same as described in Supplemental Table 1.

Localization	Ref	Alt	Name	Genotype	Polyphen	Sift	Protein Alt.
1:150240394	G	A	*APH1A*	0/1	na	na	p.Gln83X
1:220142231	G	A	*EPRS*	0/1	probably damaging	tolerated	p.Leu1486Phe
2:51254934	G	C	*NRXN1*	0/1	benign, unknown, possibly damaging	tolerated	p.Pro160Ala
2:160194196	C	G	*BAZ2B*	0/1	unknown, probably damaging	tolerated, deleterious	p.Asp1848His

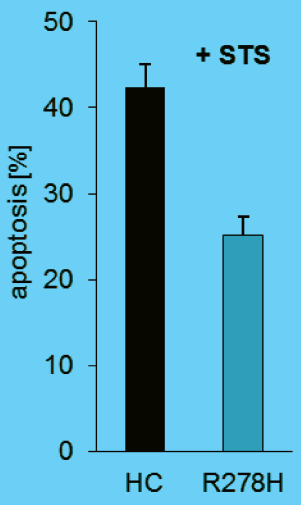
3:65415285	T	A	*MAGI1*	0/1	probably damaging	deleterious	p.Asn693Tyr
4:160264222	C	G	*RAPGEF2*	0/1	possibly damaging	tolerated	p.Leu843Val
5:666117	C	T	*TPPP*	0/1	possibly damaging	tolerated	p.Glu145Lys
5:55088486	C	G	*DDX4*	0/1	benign, possibly damaging	deleterious	p.Ile440Met
6:34214896	G	A	*C6orf1*	0/1	probably damaging	''	p.Pro17Leu
6:35911727	G	C	*SLC26A8*	0/1	possibly damaging	tolerated	p.His955Asp
8:55540031	A	G	*RP1*	0/1	benign	deleterious	p.Ser1197Gly
9:119625933	G	A	*ASTN2*	0/1	unknown, possibly damaging	deleterious	p.Arg606Trp
10:95241888	G	T	*MYOF*	0/1	possibly damaging, benign	deleterious	p.Pro22Thr
11:130784550	C	G	*SNX19*	0/1	probably damaging, benign	deleterious	p.Gly429Arg
12:130912743	G	A	*RIMBP2*	0/1	possibly damaging	deleterious	p.Thr781Met
15:74883550	G	C	*ARID3B*	0/1	probably damaging	deleterious	p.Glu314Gln
16:21063004	C	T	*DNAH3*	0/1	probably damaging	deleterious	p.Asp1409Asn
16:89788986	A	C	*ZNF276*	0/1	probably damaging	deleterious	p.Lys85Gln
17:38978767	C	T	*KRT10*	0/1	unknown	deleterious	p.Gly24Glu
17:40481624	A	G	*STAT3*	0/1	probably damaging	deleterious	p.Met394Thr
17:48616582	T	C	*EPN3*	0/1	benign	tolerated, deleterious	p.Met266Thr
19:694910	T	C	*PRSS57*	0/1	probably damaging	deleterious	p.Tyr47Cys
19:48258773	C	T	*GLTSCR2*	0/1	probably damaging	deleterious	p.Arg408Trp
19:51411877	C	T	*KLK4*	0/1	probably damaging	deleterious	p.Gly145Arg
19:59074080	A	G	*MZF1*	0/1	possibly damaging	deleterious	p.Phe522Leu

20:46265006	A	T	*NCOA3*	0/1	probably damaging	deleterious	p.Thr626Ser
21:35190673	G	A	*ITSN1*	0/1	probably damaging, unknown, benign	tolerated	p.Glu944Lys

10. Supplemental Figures



Supplemental Figure 1: FAS cell surface expression on primary T cells of patient 2 (right panel) is decreased compared to T cells from a healthy control (middle panel). A FITC conjugated FAS (CD95, BD Biosciences) antibody was used for flow cytometric detection of cell surface FAS receptor (lower right quadrant). Left panel: Isotype control. A representative experiment performed three times in duplicates is shown.



Supplemental Figure 2: *STAT3* gain-of-function mutation protects lymphocytes from staurosporine induced apoptosis. EBV transformed R278H B cells are less susceptible to apoptosis induced by staurosporine (0.5 µM). Apoptosis was measured after 24 h of treatment. Apoptosis was measured by flow cytometry using annexin V-FITC/propidium iodide staining. Mean values and standard deviations of a representative experiment of three, performed in duplicates are shown.

5.1.2 Specific antibody deficiency and autoinflammatory disease extend the clinical and immunological spectrum of heterozygous *NFKB1* loss-of-function mutations in humans

Cyrill Schipp*[1], Schafiq Nabhani*[1], Kirsten Bienemann[1], Natalia Simanovsky[2], Shlomit Kfir-Erenfeld[3], Nathalie Assayag-Asherie[3], Prasad T. Oommen[1], Shoshana Revel-Vilk[4], Andrea Hönscheid[1], Michael Gombert[1], Sebastian Ginzel[1,5], Daniel Schäfer[1], Hans-Jürgen Laws[1], Eitan Yefenof[3], Bernhard Fleckenstein[6], Arndt Borkhardt[1], Polina Stepensky*[4], and Ute Fischer*[1]

[1]Department of Pediatric Oncology, Hematology and Clinical Immunology, University Children's Hospital, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany;
[2]Department of Radiology, Hadassah Hebrew University Medical Center, Jerusalem, Israel;
[3]The Lautenberg Research Center, Hebrew University-Hadassah Medical School, Jerusalem, Israel;
[4]Pediatric Hematology Oncology and Bone Marrow Transplantation Department, Hadassah Hebrew University Medical Center, Jerusalem, Israel;
[5]Department of Computer Science, Bonn-Rhine-Sieg University of Applied Sciences, Sankt Augustin, Germany
[6]Department of Clinical and Molecular Virology, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany
CS and SN as well as PS and UF contributed equally to this work.

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Contributions of Cyrill Schipp (co-first author)

- Bioinformatical analysis (100%)
- Designed experiments (80%)
- Performed experiments (50%)
- Analyzed data (100%)
- Statistical analysis (100%)
- Manuscript writing (20%)

1. Article

The nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB1) is a master regulator of immune and inflammatory responses [1,2]. NF-κB1 belongs to the NF-κB/Rel family of transcription factors that consists of five members in humans: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA, c-Rel, and RelB. The p105 and p100 precursors are proteolytically processed by the proteasome to generate the shorter p50 and p52 isoforms. Homo- and heterodimers are formed by p50, p52 and the Rel proteins. Unstimulated, these dimeric complexes are sequestered in the cytoplasm by inhibitory IκB proteins in an inactive state. Upon stimulation, phosphorylation and subsequent degradation of IκB proteins is rapidly triggered, releasing the NF-κB/Rel complexes to enter the nucleus where they bind to DNA at κB sites and activate or repress the expression of their target genes [3].

Recently, heterozygous mutations affecting the *NFKB1* gene were identified in three human families. Haploinsufficiency of NF-κB1 was causative for combined variable immunodeficiency (CVID) characterized by recurrent infections due to immunoglobulin deficiency (pan-IgG, IgA and/or IgM).[4]

We report here on two pediatric patients from unrelated families with two novel *NFKB1* gene mutations identified by whole-exome sequencing (Figure 1A, B). Both patients had early onset of disease during their teenage years and presented in addition to hypogammaglobulinemia or selective IgA deficiency with a striking lack of specific antibodies (clinical characteristics are summarized in Table 1 and Supplemental Table 1).

Patient 1 is a now 26-year-old female who first presented with recurrent autoimmune hemolytic anemia at the age of 14. Hypogammaglobulinemia (IgG2 subclass deficiency), deficient production of specific antibodies, decreased class-switched and memory B-cells, naïve CD4-positive and regulatory T-cells, increased activated and double-negative T-cells (DNT-cells, CD4-CD8-TCRα/β+), autoimmune phenomena (hemolytic anemia, thrombocytopenia and leukopenia), lymphadenopathy, and hepatosplenomegaly observed. She developed chronic lung disease with bronchiectases, frequent respiratory tract infections and pneumonia. Infections with viral, bacterial and fungal pathogens were frequent. She suffered from intractable abdominal pain and bloody diarrhea without evidence of infection. After a liver biopsy she developed pancolitis with subsequent sepsis and multi-organ failure and was successfully resuscitated. The patient is treated with intravenous immunoglobulin. Steroids were intermittently given to reduce pulmonary

infiltrates with partial response. Mycophenolate mofetil stabilized blood counts, but pulmonary symptoms and infections remained.

To identify the genetic cause of disease whole exome sequencing was performed for the patient and her family (Supplementary Material and Methods, Supplementary Table 2). A heterozygous *de novo NFKB1* frameshift mutation was detected (Figure 1A). The *NFKB1* gene encodes two proteins: p50 and its precursor p105. The mutation led to early protein truncation (p.I47YfsX2) and decreased expression of both protein isoforms (Figure 1B, C). RNA expression was slightly reduced.

Patient 2 is a 19-year-old female from a consanguineous family (Figure 1A), who presented at the age of 11 with recurrent respiratory infections leading to chronic lung disease with bronchiectasis. She suffered from lymphadenopathy, splenomegaly, recurrent autoimmune hemolytic anemia and diarrhea. Whole exome sequencing revealed an inherited heterozygous nonsense *NFKB1* mutation (p.R157X) leading to early protein truncation and decreased p50/p105 expression (Figure 1B, D). *NFKB1* mRNA levels were low suggesting that the mutation may lead to mRNA instability and nonsense-mediated decay. Sequencing of reverse transcribed mRNA (Figure 1C, D, lower panels) indicated the absence of mutated mRNA in cells of patient 2, but not patient 1. Patient 2 had a selective IgA deficiency, IgG and IgM levels were within normal range. Similar to patient 1, she lacked specific antibodies including isohemagglutinins. Immunophenotyping demonstrated normal absolute numbers of T-, B- and NK-cells, but decreased numbers of naïve T-cells and increased levels of activated, DNT- and TCRα/β-positive T-cells. Splenomegaly and bronchiectases are shown in Figure 1E. The father and two siblings carried the same mutation, so far without clinical signs except low IgM, IgG1 and IgA levels (Supplemental Table 3). Elevated levels of IgG2, IgG3 or IgG4 may compensate for lack of IgG1 in these mutation carriers.

To analyze the impact of the mutations on NF-κB mediated signaling, we tested the response to stimuli known to trigger the canonical NF-κB pathway (Figure 2A). We treated EBV-transformed B-cells of patient 2 and healthy controls with lipopolysaccharide (LPS), TNFα or interleukin 1 β (IL-1β) and analyzed expression of *NFKB1* (itself a target of NF-κB mediated regulation). *NFKB1* expression was upregulated in control, but not in R157X-mutant cells. The lack of measurable *NFKB1* transcriptional response in the heterozygous cells suggests a more profound effect of the mutation than a mere p50/p105 dose reduction would explain. Upregulation of other target genes, such as *CFLAR*, upon activation was deficient in R157X-mutant cells (Figure 2B). To analyze the alteration of

NF-κB regulated gene expression in more detail we used predesigned real-time PCR assays that include 84 NF-κB targets (Supplemental Figure 1). The basal expression pattern of target genes was largely different in wildtype compared to R157X-mutant cells. LPS stimulation demonstrated stark differences in inducible NF-κB target gene expression. Of the NF-κB genes, *RELA* and *RELB* were similarly expressed in mutant and control cells (at baseline and stimulated), whereas *NFKB1*, *NFKB2* and *REL* were lower expressed in mutated cells and only *REL* showed inducible expression upon LPS treatment (Supplemental Figure 2). Only 12 of 79 evaluable genes showed a comparable expression pattern in wildtype and *NFKB1* mutant cells indicating compensatory regulations or independence of p50/p105 in the presence of other NF-κB proteins. When we compared primary T cells of patient 1 to two healthy controls (Figure 2C) baseline expression of all NF-κB genes was reduced and further 40 known targets were differentially regulated. Both patients demonstrated a disturbed expression and regulation of the death receptor *FAS*. Instead of downregulating *FAS* expression during infection to allow for effective immune response, LPS induced *FAS* upregulation (Supplemental Figure 1). Accordingly, in vitro stimulation of primary T-cells with recombinant FAS ligand resulted in significantly decreased cell death in the presence of LPS or TNFα in control, but not cells derived from both patients (Figure 2D). Both patients presented with clinical features similar to the FAS signaling pathway associated autoimmune lymphoproliferative syndrome (increased DNT-cells, hepatosplenomegaly, lymphadenopathy, autoimmune symptoms, low regulatory T-cells), but FAS ligand induced apoptosis was not deficient, ALPS associated gene defects were absent and infections were too frequently.

Gastrointestinal symptoms of patient 1 were reminiscent of ulcerative colitis that is acknowledged as a systemic autoimmune disease of unknown genetic cause. A candidate gene is NFKB1 [5]. Susceptibility to inflammatory bowel disease can be caused by mutant NOD2 that fails to activate NF-κB in response to bacterial infection [6]. An *NFKB1* promoter polymorphism (-94delATTG) leading to lower LPS-induced gene expression was described in inflammatory bowel disease [7].

A characteristic of both presented patients was the deficiency to develop specific antibodies. The genetic cause of specific antibody deficiency is not known, but heterozygous deletion of IkBα led to functional NF-κB haploinsufficiency and lack of specific antibody response after immunization [8]. Reduced p50 expression may specifically play a role. Mice with targeted p50 disruption were deficient in producing specific antibodies in response to vaccination [9], whereas mice lacking only the C terminal half of the p105 precursor, but expressing the N terminal p50 protein (p105-/-), were not [10].

Immunoglobulin class switch is known to be regulated by NF-κB. Mutations in the NF-κB essential modulator (NEMO) cause a class switch defective hyper-IgM syndrome in humans [12]. Decreases in IgG, IgA, IgE, but not IgM were observed in p50 knockout mice [9-11]. NF-κB regulates the expression of activation-induced cytidine deaminase (AID encoded by the *AICDA* gene), an enzyme required for DNA cleavage during class switch recombination. *AICDA*-knockout mice and patients with *AICDA* mutations are both class switch deficient. Thus, low levels of NF-κB may impair AID mediated class switch recombination [13].

Loss of p105 in the patients may play a role in the pathogenesis of infections and inflammatory disease, lymphadenopathy and splenomegaly that were also observed in the mouse model [10]. p105 knockout led to constitutive activation of p50 homodimer repressors/activators indicating that low expression of *NFKB1* may result in a severe dysregulation of the NF-κB network.

Similar to other gene defects impacting on immune system control, *NFKB1* mutations lack complete penetrance [14]. In three pedigrees with 20 individuals harboring mutant *NFKB1* alleles, the onset of disease varied from 2-64 years of age. Two gene carriers were completely healthy, while others had mild phenotypes with transient hypogammaglobulinemia or mild infections [4]. Gender may be a bias, because most clinically affected individuals were females (17 of 22 cases). In our patients, we did not detect mutations in possible modifier genes on the X chromosome (e.g. Foxp3, TLR7). Taking into account that our and most of the reported cases (18 of 20) were affected during or after puberty we may speculate that X-linked hormonal differences may contribute to the higher penetrance of the disease in females. In this line, one of our patients showed reduced numbers of regulatory T cells. The number of these cells can also be affected by hormonal fluctuations [15].

Our results broaden the phenotypic spectrum of *NFKB1* associated disease and indicate novel implications for diagnosis and treatment.

2. Acknowledgments

The authors would like to thank Katayoun Alemazkour, Bianca Killing and Monika Schmidt for excellent technical assistance. This work was funded by an intramural grant (05/2014) of the research commission of the medical faculty of the Heinrich-Heine-University Düsseldorf.

3. Tables

Table 1: Clinical and laboratory characteristics of two patients with novel NFkB1 mutations Abbreviations: AIHA, autoimmune hemolytic anemia; DNT, double negative T cells; EBV, Epstein Barr virus; HHV, human herpes virus; HPV, human papilloma virus; HSV, herpes simplex virus; IgA, immunoglobulin A; ITP, idiopathic thrombocytopenia; IVIG, intravenous immunoglobulin; RSV, Respiratory-Syncytial-Virus; SIRS, systemic inflammatory response after surgery; Staph., Staphylococcus; Strep., Streptococcus; TCR, T cell receptor; URTI, upper respiratory tract infection

	Patient 1	**Patient 2**
Mutation	p.Ile47TyrfsX2 (exon 4)	p.Arg157X (exon 7)
Sex	Female	Female
Born	1990	1997
Age	14 years (at presentation)	11 years (at presentation)
Hematology	Hepatosplenomegaly, lymphadenopathy, panhypogammaglobulinemia, specific antibody deficiency, increased DNT cells, decreased B cells, naive T cells and Tregs, SIRS after liver biopsy	Splenomegaly, selective IgA deficiency, specific antibody deficiency, increased DNT and TCRα/β+ T cells, decreased naïve T cells
Autoimmunity	AIHA, ITP, pancytopenia, autoantibodies	AIHA, ITP, leukopenia
Infection, inflammation	Recurrent pneumonias, URTI, fever, chronic otitis, mastoiditis, sinusitis, vulvovaginitis	Recurrent pneumonias, URTI
Pathogens	Recurrent viral, bacterial and candida infections (chronic EBV, recurrent Herpes zoster; *vaginal*: HPV83, HSV, Gardnerella and group B Strep., Citrobacter Braakii, Candida lusitaniae; *oral*: HSV, RSV; *blood*: EBV, HHV6; *ear*: Staph. Aureus, group A Strep.; *colon*: Adenovirus)	Streptococcus pneumonia, Haemophilus influenza, Salmonella infections
Gut disease	Pancolitis, recurrent idiopathic diarrhea, abdominal pain, bloody stools, ascites	Recurrent diarrhea
Other clinical findings	Chronic inflammatory lung disease, organ infiltration (liver, lung, spleen, kidney), nephromegaly, impaired kidney function, intermittend proteinuria, latent hypothyreosis, multiple ovarial cysts, lack of calcium and vitamin D, recurrent headaches with vertigo, numbness and paresis of left arm and hand, generalized mucositis, recurrent aphtae and painful ulcers of mouth, esophagus and genitalia	Chronic lung disease, bronchiectasis, clubbing
Treatments	IVIG, steroids, mycophenolat mofetil, antibiotics, antiviral medication, calcium, vitamin D	IVIG, steroids

Surgery	Cholecystectomy, colectomy, mastoidectomy, ulcus excision, myringotomy and tympanostomy tube insertion		
Total Immunoglobulins:			Norm
Age at examination	22	19	
IgM (mg/dL)	**24**	362	34-250
IgG (mg/dL)	**485**	1091	680-1530
IgA (mg/dL)	**<6.2**	**<5**	66-300
Specific antibodies:			
Isohemagglutinins	Anti-A1 1:4 Anti-A2 1:4 Anti-B 1:2 (blood group O Rh)	absent	
Anti-diphtheria toxoid	< 0.1 IU/ml		low protection: >0.01-0.1 protection: >0.1
Anti-tetanus toxoid	< 0.1 IU/ml		low protection: >0.01-1.0 protection: >1.0
Others		Lack of antibodies against measles, mumps, rubella, hepatitis A and B	

4. Figures

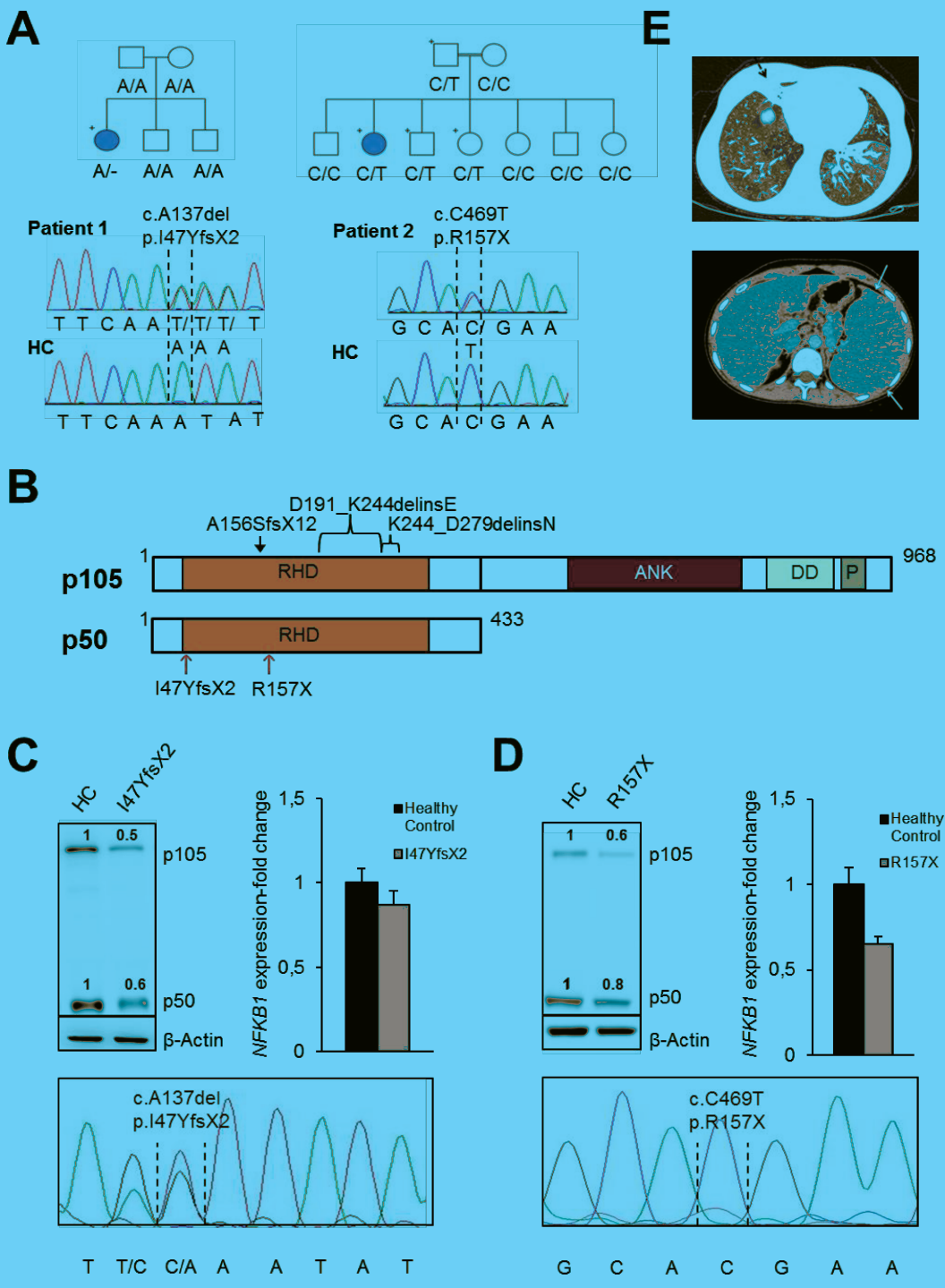

Figure 1: Two novel heterozygous *NFKB1* mutations detected in two families decrease the protein levels of p105 and p50. (A) Upper left: Whole-exome sequencing identified a heterozygous *NFKB1* mutation (A/-) in patient 1. The patient is the only carrier of the mutation in the family pedigree (indicated by "+") and the only diseased family member (indicated by a filled circle). Lower left: Capillary sequencing using genomic DNA confirmed an *NFKB1* frameshift mutation (c.A137del, p.I47YfsX2) in patient 1. Representative chromatograms of patient 1 and a healthy control are shown. Upper right: Patient 2 descended from consanguineous parents and harbors an inherited heterozygous *NFKB1* mutation (C/T). The patient is the only diseased family member. The father and two siblings carry the same mutation but are not affected. Sanger sequencing of *NFKB1* confirmed the heterozygous missense mutation (c.C469T, p.R157X) in patient 2.

Representative chromatograms are shown. **(B)** Schematic drawing of the proteins p105 and p50 and their domains that are both encoded by the *NFKB1* gene. The mutations in the Rel homology domain (RHD) identified in the two patients (red arrows) lead to early protein truncation of both proteins. Previously reported heterozygous germline mutations associated with CVID are indicated on top (black arrow and brackets). ANK, ankyrin repeats; DD, death domain; P, PEST domain enriched for proline (P), glutamic acid (E), serine (S), and threonine (T) residues. **(C, D)** Expression of p105 and p50 proteins is decreased in the affected patients. **(C)** Primary T cells of patient 1 and healthy controls were activated by PHA in the presence of IL-2. Protein and RNA extracts were prepared. Western blot analysis was carried out employing a specific p105/p50 antibody using β-actin as a loading control (left panel). *NFKB1* mRNA expression was measured by real-time PCR (right panel). The fold change in cells of the patient compared to a representative healthy control is shown, GAPDH and β-actin expression were used as internal standards. Mean values of representative experiments performed in triplicates and corresponding SDs are shown. Sanger sequencing using reverse transcribed mRNA of the patient demonstrates the presence of mutated *NFKB1* transcripts (lower panel). **(D)** EBV transformed B cells of patient 2 were used for protein and RNA extraction. Analysis of *NFKB1* protein and RNA expression was carried out as described in (C). Capillary sequencing of cDNA from patient 2 fails to detect the *NFKB1* mutation indicating that the mutation leads to mRNA instability (lower panel). **(E)** Upper panel: Axial high resolution chest CT image of patient 2 at the level of lung bases demonstrating multiple areas of bronchiectases (white arrows) and consolidation with atelectatic component surrounding bronchiectases in the right middle lobe (black dashed arrow). Mosaic pattern of perfusion of the lung parenchyma is noted, with multiple areas of low attenuation in the right low lobe (arrowheads). Lower panel: Axial CT image of patient 2 at the level of the upper abdomen demonstrating the enlarged spleen.

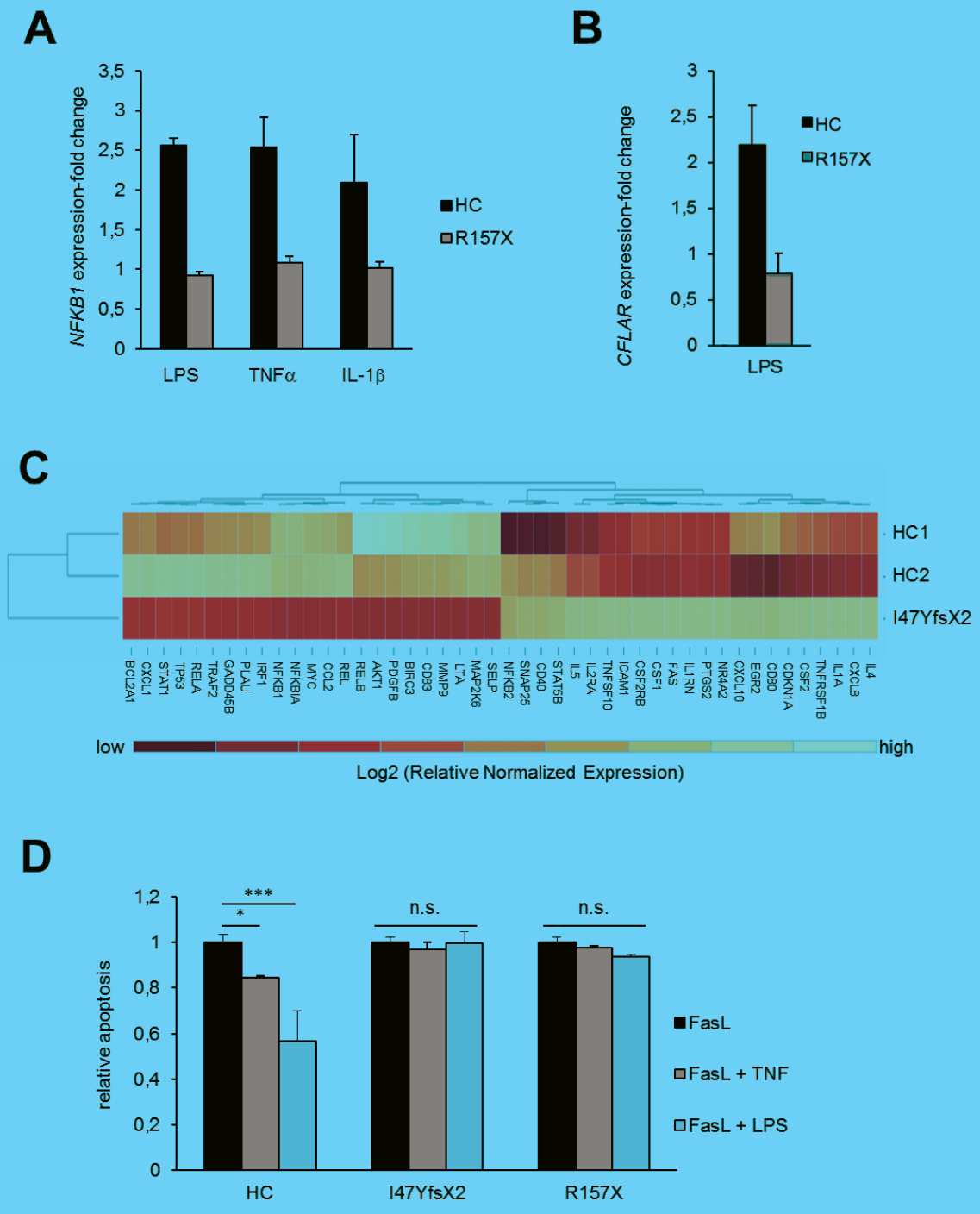

Figure 2: NF-κB mediated signaling is affected in the patients. (A) Upregulation of *NFKB1* mRNA expression in response to NF-κB activating stimuli is deficient in *NFKB1* mutated cells. EBV immortalized B cells of patient 2 and of a representative healthy control were treated for 16 h with LPS (5 µg/ml), TNFα (50 ng/ml) or IL-1β (10 ng/ml), respectively. Fold change of *NFKB1* mRNA expression in untreated compared to treated samples was determined by real-time PCR. The relative expression was normalized to the respective untreated controls (=1). GAPDH and β-actin expression were used as internal standards. Mean values of representative experiments performed in triplicates and corresponding SDs are shown. **(B)** Expression of the NF-κB target gene *CFLAR* (synonymous for cFLIP) is not induced by LPS in *NFKB1* mutated patient cells. EBV immortalized B cells of patient 2 and healthy control cells were treated for 16 h with LPS (5 µg/ml). Fold change of mRNA expression of *CFLAR* (synonymous for cFLIP) was determined by real-time PCR as described in Figure 1C. **(C)** Differential expression of NF-κB target genes in *NFKB1* mutated primary T cells of patient 1 and two healthy wildtype controls. Baseline expression of a panel of NF-κB target genes was analyzed by real-

time PCR using predesigned arrays (NF-κB signaling targets RT2 Profiler PCR arrays, Qiagen, Hilden, Germany). Mean values of two independent assays are shown. High gene expression is indicated in light color, low gene expression in dark red. **(D)** LPS does not protect *NFKB1* mutant primary T cells from apoptosis. Primary T cells of patient 1 and 2 and healthy controls were activated by PHA and IL2. Cells were treated with 100 ng/ml recombinant FAS ligand to induce apoptosis in the presence or absence of 100 ng/ml LPS or 10 ng/ml TNFα. Apoptosis was determined by flow cytometric measurement of phosphatidylserine exposure indicated by binding of annexin V-FITC. Dead cells were detected with propidium iodide. Significance was tested using 2way Anova (* p<0,05; ***p<0,001).

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6. Supplementary Methods

All experiments were performed after obtaining written informed consent from the patients and their families and were approved by the Ethical Review Boards of Hadassah, the Israeli Ministry of Health and the local Ethics committee of the University of Duesseldorf.

Whole-exome sequencing and bioinformatic analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA for the patient and family members using the SureSelect Human All Exon V5+UTR kit (Agilent, Santa Clara, CA). 100 bp paired-end read sequencing was performed on a HiSeq2500 (Illumina, San Diego, CA) as recommended by the manufacturer.

Briefly, sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA.[1] Sequencing data was converted using Samtools.[2] Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor[3], based on the Ensemble database (v70) (www.ensembl.org). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

Validation and analysis of the NFKB1 sequence variations using genomic and cDNA

Validation of the nucleotide variations in the *NFKB1* gene were performed by PCR/Sanger sequencing using genomic DNA from the patients and their family members. The following primers were used: *NFKB1*-I47fsX2 (forward: 5' ACTTATTGTGGTTCGCTAAACTCG 3', reverse: 5' AGGACAGTGTGAACAATGAGTAAG 3'), *NFKB1*-R157X (forward: 5' CATGTAGCCCCAAGAGATTTG 3', reverse: 5' GGGTAGAGACTGGAAGGG 3'). DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 µM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 seconds at 98°C followed by 30 cycles of 7 seconds at 98°C, 23 seconds at 60°C, 30 seconds at 72°C and final extension of 10 minutes at 72°C. Sanger sequencing was carried out by the core facility of the Heinrich-Heine-University (BMFZ). Nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

To analyze the presence of mutated transcripts within the mRNA pool, total RNA was isolated from primary or EBV transformed cells derived from the patients and healthy controls using the RNeasy Blood kit (Qiagen, Hilden, Germany). cDNA was synthesized

employing the QuantiTect Reverse Transcription kit (QIAGEN). Sequencing of cDNA was performed after PCR amplification using the same conditions as described above. The following primers were employed: *NFKB1*-I47fsX2 (forward: 5'-GATCCATATTTGGGAAGGCCTGAAC-3', reverse: 5'-CAACTGAACAATAACCTTTGCTGGTC-3'), *NFKB1*-R157X (forward: 5'-ATATCCACCTGCATGCCCAC-3', reverse: 5'-GGTCCATCTCCTTGGTCTGC-3').

Isolation and cultivation of primary T cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells were isolated using density gradient centrifugation and cultured in medium consisting of RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% FCS and 100 µg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach, Germany). For the first 4 days, cells were activated by addition of 7 µg/ml phytohemagglutinine (PHA, Life Technologies).

Transformation of primary B lymphocytes

A B cell line was generated by transformation with Epstein-Barr virus (EBV) (ATCC, Wesel, Germany) as described previously[1] and cultured in RPMI1640 supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies).

Immunoblotting

Cells were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 8-15% polyacrylamide gels, transferred to nitrocellulose membranes and detected by chemiluminescence (GE Healthcare, Freiburg, Germany). The following primary antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO), NF-κB p105/p50 (Cell Signaling, Frankfurt am Main, Germany).

Real-time PCR and NF-κB target gene expression arrays

Total RNA was isolated from primary and EBV transformed cells derived from the patients and healthy controls using the RNeasy Blood kit (Qiagen, Hilden, Germany). cDNA was synthesized employing the QuantiTect Reverse Transcription kit (QIAGEN) and Real-time PCR was performed using QuantiTect primers for *GAPDH*, *ACTB*, *NFKB1, CFLAR* (QIAGEN) and the Power SYBR Green PCR Mastermix (Applied Biosystems) according to the manufacturer's recommendations. To assess differences in NF-κB target gene expression,

predesigned real-time PCR arrays were employed (NF-κB pathway PrimePCR Replica Panels were used and analyzed using the PrimePCR Analysis software tool according to the recommendations of the manufacturer (Bio-Rad, Munich, Germany); NF-κB signaling targets RT^2 Profiler PCR arrays and analysis software, Qiagen, Hilden, Germany). Differential expression of NF-κB target genes in *NFKB1* mutated transformed B cells derived from patient 2 and wild type controls were analyzed by real-time PCR arrays after 3 h of LPS stimulation (5 µg/ml). Expression of NF-κB target genes in primary T cells of patient 1 and healthy wildtype controls was analyzed by real-time PCR after overnight stimulation with LPS (500 ng/ml). Mean values of two independent assays are shown.

Immunophenotyping

Immunophenotyping was carried out in the clinical setting according to standard protocols. In addition immunophenotyping for ALPS-like features was carried out employing the following antibodies: mouse anti-human CD3 (clone UCHT1, BD Biosciences, Heidelberg, Germany), mouse anti-human TCR αβ (clone T10B9.1A-31, BD Biosciences), mouse anti-human CD4 (clone VIT4, Miltenyi), mouse anti-human CD8 (clone BW135/80, Miltenyi), anti-B220, anti-HLA-DR, anti-CD27, anti-CD19, and anti-CD25 (all from BD Biosciences) and anti-CD45R (Beckman Coulter, Krefeld, Germany).

Apoptosis assay

Activated primary T cells from the patients and healthy controls were plated in 96 well plates at a density of 10^6 cells/ml and stimulated with recombinant SuperFasLigand (100 ng/ml, Enzo Life Sciences, Lörrach, Germany) in the presence or absence of LPS (100 ng/ml, Salmonella spec., supplied by Sigma-Aldrich, Deisenhofen, Germany) or TNFα (10 ng/ml, Sigma-Aldrich). After 24 h cells were stained with annexin V-FITC (BD Biosciences) and propidium iodide (PI, Sigma-Aldrich) and apoptosis was measured employing a FACSCalibur according to the manufacturer's suggestions (BD Biosciences).

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8. Supplemental Tables

Supplemental Table 1: Clinical and laboratory characteristics of the patients (Values varying significantly from norm values are indicated in bold. Ranges indicate variations between multiple measurements done. Abbreviations: nd, not done; na, not available (i.e. not done on presentation, the patient receives IVIG)

	Patient 1	Patient 2	Norm Values
NFKB1 mutation	**p.I47YfsX2**	**p.R157X**	
Age at examination	26	19	
Immunoglobulin levels:			
IgM (mg/dL)	24	362	40-230
IgG (mg/dL)	**485**	**1091**	**700-1600**
IgG1 (mg/dL)	342	na	420-1100
IgG2 (mg/dL)	**37.4**	**na**	**140-480**
IgG3 (mg/dL)	114	na	35-128
IgG4 (mg/dL)	**<0.3**	**na**	**7-48**
IgA (mg/dL)	<6.2	<5	70-400
Flow cytometry (of gated lymphocytes):			
CD3+ (T cells) (%)	57-91	87.2	55-84
CD4+/CD3+ (T helper) (%)	**40-65**	**54.1**	**31-60**
CD8+/CD3+ (T suppressor) (%)	13-34	29.3	13-41
TCRα/β+ (% of CD3+)	**0.3-1.2**	**7.2**	**3-10**
DNT cells (TCRα/β+/CD4-/CD8-) (% of CD3+)	1.1-4.6	3-20	<2.5
B220+ (% of DNT)	**nd**	**81.57**	**8-40**
CD25+ (% of CD3+)	0.2-0.9	1.1	<18
HLA-DR+/CD3+ (%) (activated T-cells)	**8.2-17.6**	**33.4**	**<10**
Regulatory T cells (CD25+/FoxP3+) (% of CD4+)	0.18	1.7	1-2
CD25+ (% of CD4+)	**0.1-3.5**	**3.3**	**>2.5-12.5**
CD45RA+ (% of CD4+) (naïve CD4 cells)	7-9	9.8	40-65
CD45RO+ (% of CD4+) (memory CD4 cells)	**91-93**	**90.5**	**25-52**
CD45RA+ (% of CD8+) (naïve CD8 cells)	31-58	62.6	6-100
CD45RO+ (% of CD8+) (memory CD8 cells)	**42-69**	**37.3**	**14-98**
CD56+/CD3- (%, natural killer cells)	4-8	6.4	5-27
CD19+ (%)	**4-11**	**3.9**	**6-25**
IgD+/CD27- (% of CD19+) (naïve B cells)	70-79	39.9	75-86
IgD+/CD27+ (% of CD19+) (intermediate B cells)	**6-12**	**31.2**	**4-10**
IgD-/CD27+ (% of CD19+) (memory B cells)	0.44-5	20.3	3-10
HLA A, B, C+ (%)	**95-100**	**96.8**	**≈100**

Supplemental Table 2: Candidate genes of patient 1 identified by whole-exome sequencing Abbreviations: Ref., reference sequence; Alt., altered sequence; np, no prediction provided by the tool. Polyphen and Sift are tools that predict detrimental effects of nucleotide mutations on protein function based on sequence conservation and protein structure.[5,6]

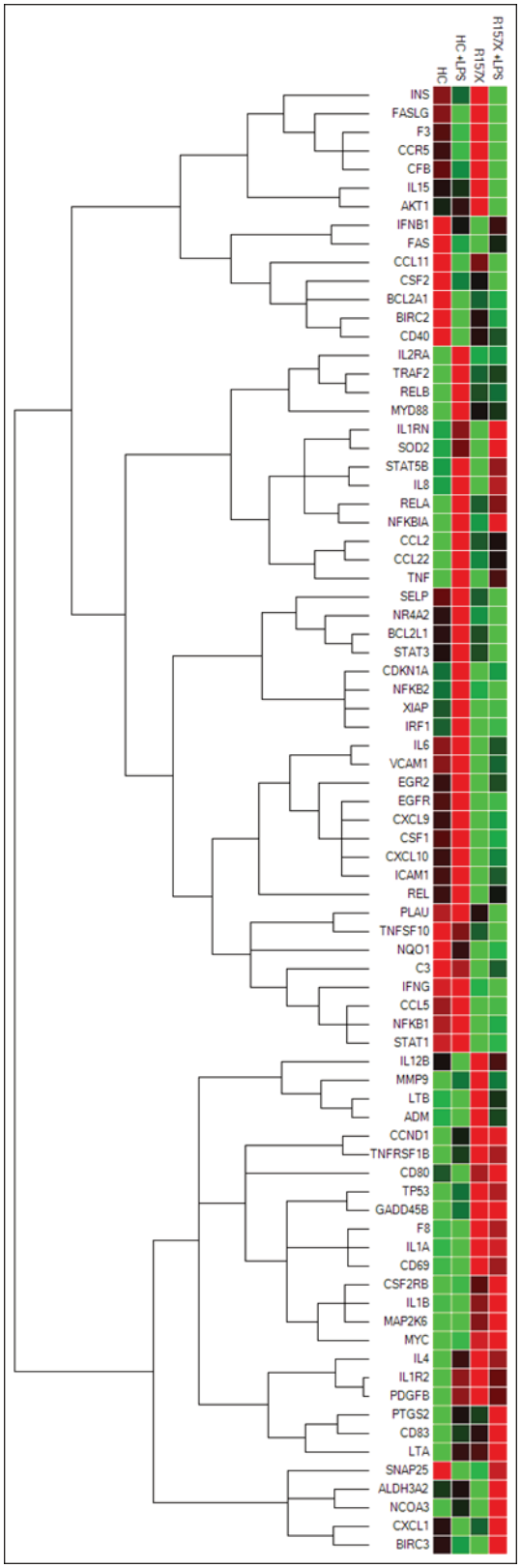
Localization	Ref.	Alt.	Gene	Genotype	Mutation	Protein
4:103455019	CA	C	*NFKB1*	0/1	frameshift	p.Ile47TyrfsX2
5:115202417	TAAGA	T	*AP3S1*	0/1	frameshift	p.Lys41GlufsX9
10:117075087	T	TGG	*ATRNL1*	0/1	frameshift	p.Cys961GlyfsX22
10:126678133	TG	T	*CTBP2*	0/1	frameshift	p.Gln431SerfsX36
11:76895792	T	G	*MYO7A*	0/1	stop lost	p.X1179GlyextX6
15:28518046	G	A	*HERC2*	0/1	missense	p.Thr302Met
17:34091040	T	A	*C17orf50*	0/1	missense	p.Leu10Met
17:45247388	AT	A	*CDC27*	0/1	frameshift	p.Ile91SerfsX54

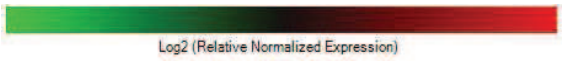
Supplemental Table 3: Clinical and laboratory characteristics of the asymptomatic mutation carriers in the family of patient 2 ND*, not done on presentation, the patient receives IVIG treatment.

	Patient 2	Father	Brother	Sister	Norm
NFKB1 mutation	R157X	R157X	R157X	R157X	
Age at examination (years)	19	51	15	22	
Immunoglobulines levels:					
IgM (mg/dL)	362	**22**	**21.5**	**31.1**	34-400
IgG (mg/dL)	1091	837	**596**	**653**	680-1530
IgG1 (mg/dL)	ND*	**164**	**13**	**34**	420-1100
IgG2 (mg/dL)	ND*	**651**	**498**	**531**	140-480
IgG3 (mg/dL)	ND*	**198**	108	**138**	35-128
IgG4 (mg/dL)	ND*	56	37.4	57	7-48
IgA (mg/dL)	**<5**	91	**48**	**11.3**	66-407
Flow cytometry (of gated lymphocytes):					
CD3+ (T cells) (%)	87.2	65.4	66.9	73.6	55-84
CD4+/CD3+ (T helper) (%)	54.1	42.7	26.9	36.3	31-60
CD8+/CD3+ (T suppressor) (%)	29.3	18.8	25.4	31.1	13-41
TCRγδ+ (% of CD3+)	7.2	2.1	**19.5**	3.6	3-10
DNT cells (TCRα/β+/CD4-/CD8-)	**3.1**	2.1	1.4	2.0	<2.5

(% of CD3+)					
CD25+ (% of CD3+)	1.1	8.9	3.7	3.7	<18
HLA-DR+/CD3+ (active T-cells) (%)	**33.4**	8.8	**11.4**	9.4	<10
Regulatory T cells (CD25+/FoxP3+) (%of CD4+)	1.7	1.0	2.0	1.5	1-2
CD25+ (% of CD4+)	3.3	10.5	7.2	6.1	>2.5-12.5
CD45RA+ (% of CD4+) (naïve CD4 cells)	**9.8**	43.2	41.8	43.4	40-65
CD45RO+ (% of CD4+) (memory CD4 cells)	**90.5**	55.8	56.4	55.1	25-52
CD45RA+ (% of CD8+) (naïve CD8 cells)	62.6	66.3	60.1	75.9	6-100
CD45RO+ (% of CD8+) (memory CD8 cells)	37.3	33.8	40.1	24.2	14-98
CD56+/CD3- (% natural killer cells)	6.4	10.4	14.2	5.3	5-27
CD19+ (%)	**3.9**	19.1	12.0	14.5	6-25
IgD+/CD27- (% of CD19+) (naive B cells)	**39.9**	82.5	81.0	**57.9**	75-86
IgD+/CD27+ (% of CD19+) (intermediate B cells)	**31.2**	6.0	9.9	**30.7**	4-10
IgD-/CD27+ (% of CD19+) (memory B cells)	**20.3**	8.1	4.2	6.0	3-10
HLA A, B, C+ (%)	96.8	100.0	99.9	99.8	≈100

9. Supplemental Figures





Supplemental Figure 1: NF-κB mediated response to lipopolysaccharide (LPS) is affected in the patients. Differential expression of NF-κB target genes in wildtype and *NFKB1* mutated cells. Expression of a panel of NF-κB target genes was analyzed by real-time PCR after 3 h of LPS stimulation (5 µg/ml) using predesigned NF-κB pathway PrimePCR Replica Panels (Bio-Rad, München, Germany). Mean values of two independent assays are shown. High gene expression is indicated in red, low gene expression in green.

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VII Appendix

7.1 Abbreviations

AD	Autosomal dominant
AICDA/AID	Activation induced cytidine deaminase
AIHA	Autoimmune hemolytic anemia
ALPS	Autoimmune lymphoproliferative syndrome
ANA	Anti-nuclear antibodies
ANC	Absolute neutrophil count
ANK	Ankyrin repeat
BAD	BCL2 associated agonist of cell death
BAK	BCL-2 associated antagonist killer
BAX	BCL2 associated X
BCL	B-cell lymphoma
BH	BCL-2 homology domain
BID	BH3 interacting-domain death agonist
BIM	BCL-2 interacting mediator of cell death
CADD	Combined annotation dependent depletion
CARD	Caspase activation and recruitment domains
CASP	Cysteine-aspartic proteases
CBC	Complete blood cells
CD	Cluster of differentiation
CEDS	Caspase-8 deficiency state
CFLAR	CASP8 and FADD like apoptosis regulator
CRP	C-reactive protein
CT	Computed tomography
CTLA	Cytotoxic T-lymphocyte associated protein
CVID	Common variable immunodeficiency
Cyt-c	Cytochrome C
DALD	Dianzani autoimmune lymphoproliferative disease
DD	Death domain
DISC	Death-inducing signaling complex
DLBCL	Diffuse large B-cell lymphoma
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNT	Double negative T-cell
EBV	Epstein-Barr virus
EDTA	Ethylendiamintetraessigsäure
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal–regulated kinases
ESR	Erythrocyte sedimentation rate
FADD	FAS-associated death domain
FASLG	FAS ligand
FCS	Fetal calf serum
FERM	Four point one protein, ezrin, radixin and moesin

FITC	Fluorescein isothiocyanate
FLIP	FLICE-inhibitory protein
GAPDH	Glycerinaldehyd-3-phosphat-dehydrogenase
GOF	Gain-of-function
GRR	Glycine rich region
HC	Healthy control
HDAC	Histone-deacetylase
HEK	Human embryonic kidney
HgB	Hemoglobin
HHV	Human herpes virus
HOIL	Heme-oxidized IRP2 ubiquitin ligase
HOIP	HOIL-1-interacting protein
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinases
IL	Interleukin
IL-1R	Interleukin-1 receptor
ITP	Immune-mediated thrombocytopenia
IκB	Inhibitor of κB
JAK	Janus kinase
LGL	Large granular lymphocytic
LOF	Loss-of-function
LPS	Lipopolysaccharides
LRBA	LPS responsive beige-like anchor
MCL	Induced myeloid leukemia cell differentiation
MMF	Mycophenolate mofetil
mTOR	Mammalian target of rapamycin
NBD	NEMO binding domain
NEMO	NF-kappa-B essential modulator
NFKB/NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEST	Rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PET	Positron emission tomography
PHA	Phytohaemagglutinin
PI	Propidium iodide
PIAS	Protein inhibitors of activated STAT
PKC	Protein kinase C
PPIA	Peptidylprolyl isomerase A
qRT-PCR	Quantitative real-time polymerase chain reaction
RALD	RAS-associated autoimmune leukoproliferative disease

RAS	Rat sarcoma
RF	Rheumatoid factor
RHD	Rel-homology domain
RSV	Respiratory-syncytial-virus
S3I	STAT3 inhibitor
sFASLG	Soluble FAS ligand
SH2	Src homology 2
SIRS	Systemic inflammatory response after surgery
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
STS	Staurosporine
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPL	Tumor progression locus
TRAIL	TNF-related apoptosis inducing ligand
Treg	Regulatory T-cell
URTI	Upper respiratory tract infections
WBC	White blood cell
WT	Wildtype
XLP	X-linked lymphoproliferative syndrome

7.2 Nomenclature

Human gene:	uppercase, italic (e.g. *FAS*)
Human protein:	uppercase (e.g. FAS)
Murine gene:	first letter uppercase, italic (e.g. *Fas*)

7.3 Curriculum Vitae

Personal Data:

Name:	Cyrill Schipp
Date of Birth:	08. December 1986 in Düsseldorf
Address:	Teplitzer Str. 17, 40231 Düsseldorf

Professional Career:

Since 10/2013	Research Associate, PhD Student Department of Paediatric Oncology, Haematology and Immunology University Hospital Düsseldorf, Düsseldorf, Germany
07/2006 – 05/2007	Civil Service, Psychiatric nursing LVR-Clinic Langenfeld, Langenfeld, Germany

Education:

10/2007 - 04/2013	Biochemistry, Masters of Science Ruhr University Bochum, Bochum, Germany
08/1997 - 06/2006	Abitur Gymnasium an der Koblenzer Straße, Düsseldorf, Germany

List of Publications:

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***STAT3* gain-of-function mutations associated with autoimmune lymphoproliferative syndrome like disease deregulate lymphocyte apoptosis and can be targeted by BH3 mimetic compounds**

Nabhani, S*, **Schipp, C***, Miskin H, Levin C, Postovsky S, Dujovny T, Koren A, Harlev D, Bis AM, Auer F, Revel-Vilk S, Keller B, Warnatz K, Gombert M, Ginzel S, Borkhardt A, Stepensky P*, Fischer U* (*shared authorship)

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Haematologica 2016

Specific antibody deficiency and autoinflammatory disease extend the clinical and immunological spectrum of heterozygous *NFKB1* loss-of-function mutations in humans.

Schipp C*, Nabhani S*, Bienemann K, Simanovsky N, Kfir-Erenfeld S, Assayag-Asherie N, Oommen PT, Revel-Vilk S, Hönscheid A, Gombert M, Ginzel S, Schäfer D, Laws HJ, Yefenof E, Fleckenstein B, Borkhardt A, Stepensky P, Fischer U (*shared authorship)

Talks and posters:

San Diego, December 2-6, 2016	ASH Annual Meeting and Exposition (ASH)
	Poster
Leipzig, April 6-10, 2016	International Congress on Autoimmunity
	Poster
Genf, October 6-10, 2015	European Cell Death Organization (ECDO)
	Poster and Talk

Languages:

German:	Native language
English:	Fluent

7.4 Papers

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STAT3 gain-of-function mutations associated with autoimmune lymphoproliferative syndrome like disease deregulate lymphocyte apoptosis and can be targeted by BH3 mimetic compounds

Schafiq Nabhani [a,1], Cyrill Schipp [a,1], Hagit Miskin [b,2], Carina Levin [c,2], Sergey Postovsky [d], Tal Dujovny [c], Ariel Koren [c], Dan Harlev [b], Anne-Marie Bis [a], Franziska Auer [a], Baerbel Keller [e], Klaus Warnatz [e], Michael Gombert [a], Sebastian Ginzel [a,f], Arndt Borkhardt [a], Polina Stepensky [g,3], Ute Fischer [a,*,3]

[a] Department of Pediatric Oncology, Hematology and Clinical Immunology, University Children's Hospital, Medical Faculty, Heinrich-Heine-University Düsseldorf, Germany
[b] Pediatric Hematology Unit, Shaare Zedek Medical Center, Jerusalem, Israel
[c] Pediatric Hematology Unit, Emek Medical Center, Afula, Israel
[d] Department of Pediatric Oncology/Hematology Meyer Children's Hospital Rambam Health Care, Haifa, Israel
[e] Center for Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany
[f] Department of Computer Science, Bonn-Rhine-Sieg University of Applied Sciences, Sankt Augustin, Germany
[g] Department of Bone Marrow Transplantation, Hadassah Hebrew University Medical Center, Jerusalem, Israel

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ABSTRACT

Autoimmune lymphoproliferative syndrome (ALPS) is typically caused by mutations in genes of the extrinsic FAS mediated apoptotic pathway, but for about 30% of ALPS-like patients the genetic diagnosis is lacking. We analyzed 30 children with ALPS-like disease of unknown cause and identified two dominant gain-of-function mutations of the Signal Transducer And Activator Of Transcription 3 (STAT3, p.R278H, p.M394T) leading to increased transcriptional activity. Hyperactivity of STAT3, a known repressor of FAS, was associated with decreased FAS-mediated apoptosis, mimicking ALPS caused by FAS mutations. Expression of BCL2 family proteins, further targets of STAT3 and regulators of the intrinsic apoptotic pathway, was disturbed. Cells with hyperactive STAT3 were consequently more resistant to intrinsic apoptotic stimuli and STAT3 inhibition alleviated this effect. Importantly, STAT3-mutant cells were more sensitive to death induced by the BCL2-inhibitor ABT-737 indicating a dependence on anti-apoptotic BCL2 proteins and potential novel therapeutic options.

1. Introduction

The Canale-Smith or autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of disturbed apoptosis [1–3]. This physiological form of cell death is of fundamental importance for the regulation of cell numbers in the immune system [4]. In human cells apoptosis is conveyed *via* two major pathways. In the extrinsic pathway, cell surface death receptors (FAS, TRAIL-R1/2 and TNF-R1) are activated upon binding of their cognate death ligands (FAS-L, TRAIL, TNFα) [5]. Cell death is instantly initiated by intracellular recruitment of adapter proteins and inactive procaspase-8 and -10 into a death-inducing signaling complex that mediates the activation of the caspases. A caspase cascade is triggered by cleavage mediated activation of downstream executioner caspases. Cellular proteins are proteolyzed leading to the death of so-called type I cells (*e.g.* activated T cells). In most cells, however, the amount of death receptor activated caspases is insufficient to induce cell death (type II cells). In these cells, the signal needs to be amplified by the intrinsic mitochondria mediated pathway through cleavage of the BCL-2 protein BID by caspase-8 or -10. This leads to the formation of active truncated BID (tBID) that translocates to the mitochondria and activates the pro-apoptotic BCL-2 family members BAX and BAK1 to mediate the release of cytochrome *c* from the mitochondria into the cytosol. This triggers the assembly of the apoptosome and subsequent activation of effector caspases and cell death execution. The intrinsic apoptotic pathway can also be initiated independent of the extrinsic pathway by multiple signals, including cytokines and cellular stress. Members of the BCL2 family regulate each other through complex bimolecular interaction networks and function together as a rheostat, controlling the sensitivity of cells to apoptotic stimuli [6].

All members of the BCL2 family contain one to four conserved sequence motifs called the BCL2 homology (BH1–4) domains. Among

* Corresponding author at: Department of Pediatric Oncology, Hematology and Clinical Immunology, Center for Child and Adolescent Health, Heinrich Heine University Düsseldorf, Moorenstr. 5, 40225 Düsseldorf, Germany.
E-mail address: ute.fischer@med.uni-duesseldorf.de (U. Fischer).
[1] SN and CS contributed equally as first authors.
[2] HM and CL contributed equally as second authors.
[3] PS and UF contributed equally as last authors.

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the pro-apoptotic members of the BCL2 family, there are the so-called BH3-only proteins (including BID and BIM) that contain only the single BH3 domain [7]. The BH3 domain comprises a 9-amino acid amphipathic α-helix that binds to a hydrophobic pocket of BCL2-like anti-apoptotic proteins (including BCL2 and BCL-XL). In this manner, pro-apoptotic BH3 only proteins are kept inactive. A class of small molecule drugs (including ABT-737 [8]) mimics BH3-domain proteins and aims at disrupting this complex, thereby sensitizing cells to apoptosis.

In 70% of patients the genetic causes of ALPS are germline or somatic mutations in the genes of the extrinsic apoptotic pathway encoding the Fas receptor (*FAS*), its ligand (*FASLG*) or caspase-10 (*CASP10*) [3]. Defective death receptor signaling results in the accumulation of lymphocytes and failure to remove auto-reactive lymphocytes. As a consequence ALPS patients characteristically show enlargement of lymph nodes, spleen and liver and increased numbers of peripheral, otherwise rare terminally differentiated, activated, double-negative T lymphocytes that do not express the surface markers CD4 and CD8, but have a functional alpha/beta T-cell receptor (so called α/β-DNT cells). Although lymphoproliferation is initially non-malignant, ALPS patients have an increased risk of developing B and T cell malignancies (in particular lymphomas) early in life caused by inappropriately surviving lymphocytes with oncogenic potential [9,10]. Autoimmune disease in ALPS patients is mainly caused by B lymphocytes and directed towards blood cells. Increased numbers of B cells producing autoreactive antibodies cause various autoimmune problems: clinically significant hemolytic anemia, thrombocytopenia with bleeding tendency, autoimmune neutropenia and risk of bacterial infection. Other organs such as skin, liver, kidney, endocrine or nervous system may be affected by autoimmunity [11].

The signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family that controls apoptosis, proliferation and cell growth by regulation of gene expression in response to extracellular cytokines, IFNγ or growth hormones [12]. Somatic gain-of-function (GOF) mutations of *STAT3* cause lymphoproliferative neoplasms, aplastic anemia, and myelodysplastic syndrome [13–15], whereas germline *STAT3* gain-of-function mutations have been reported in patients suffering from recurrent infections, multi-organ autoimmunity, hypogammaglobulinemia and lymphoproliferation indicating a potential overlap of phenotype with ALPS disease [16–18]. The underlying molecular basis for lymphoproliferation in *STAT3* GOF caused disease is not yet understood. Due to this lack of knowledge and the lack of clinically approved STAT3 inhibitors, targeted treatment is not yet available.

2. Material and methods

2.1. Patients, relatives and healthy controls

Patients, relatives and healthy controls were enrolled in this study after obtaining written informed consent. All experiments were approved by the Ethical Review Boards of the Hadassah Hebrew University, the Israeli Ministry of Health and the University Hospital Düsseldorf. Whole-exome sequencing of DNA derived from peripheral blood was performed on a HiSeq2000 (Illumina, San Diego, CA).

2.2. Sanger sequencing of germline and somatic mutations in classical ALPS genes

To exclude germline or somatic variations in classical ALPS associated genes as genetic cause of the disease we amplified exons including exon/intron borders of *FAS*, *FASLG* and *CASP10* by PCR and carried out Sanger sequencing as described earlier [19].

2.3. Whole-exome sequencing and bioinformatic analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA for the patient and family members using the SeqCap EZ Exome Library 2.0 kit (Roche/Nimblegen, Madison, WI). 100 bp single-read sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA) essentially as described [20]. Sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA. Sequencing data was converted using Samtools. Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor, based on the Ensemble database (v70). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

2.4. Validation of STAT3 sequence variation

Validation of the nucleotide variations in the *STAT3* gene were performed by PCR/Sanger sequencing using genomic DNA from the patients and family members. The following primers were used: STAT3-R278H (forward: 5′ CCTTGTTCTTATTGTAGTGGTCTCC 3′, reverse: 5′ AAAGAGAAGATGGGCTCACG 3′), STAT3-M394T (according to [17]) (forward: 5′ CTTCATCCTCCGGCTACTTG 3′, reverse: 5′ GAGCTCCTCCCACATACCAA 3′). DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 μM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 s at 98 °C followed by 30 cycles of 7 s at 98 °C, 23 s at 60 °C, 30 s at 72 °C and a final extension of 10 min at 72 °C. Sanger sequencing was carried out by a core facility (BMFZ, University Duesseldorf, Germany). The nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

2.5. Isolation and cultivation of mononuclear cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells were isolated using density gradient centrifugation and cultured in medium consisting of RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% fetal calf serum (FCS) and 100 μg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach, Germany). For the first 4 days, cells were activated by addition of 7 μg/ml phytohemagglutinine (PHA, Life Technologies).

2.6. Transformation of primary B lymphocytes

Cell lines of R278H mutant STAT3 B cells and healthy controls were generated by transformation with Epstein-Barr virus (EBV) (ATCC, Wesel, Germany) as described previously [21] and cultured in RPMI1640 supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies).

2.7. Immunoblotting

Cells treated with IL6 (25 ng/ml, Miltenyi) for 24 h or left untreated were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 8–15% polyacrylamide gels, transferred to polyvinylidene fluoride membranes and detected by chemiluminescence (GE Healthcare, Freiburg, Germany). The following primary antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO), STAT3 (R&D Systems, Wiesbaden, Germany), phospho-STAT3 (Tyr705, Cell Signaling, Frankfurt am Main, Germany), BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA), BCL-XL (Cell Signaling), tubulin-HRP (Cell Signaling).

2.8. Luciferase reporter assays

STAT3 was cloned into the pMC plasmid [22] and a hygromycin expression cassette was added. STAT3 variants (R278H, K392R, M394T) were generated by site-directed mutagenesis employing the Q5 site-directed mutagenesis kit as recommended by the manufacturer (NEB). The nucleotide sequence was confirmed by Sanger sequencing on an ABI 3130 Genetic Analyzer (Applied Biosystem, Carlsbad, CA). Stable human embryonic kidney (HEK) 293 cell clones expressing STAT3 wildtype or mutant constructs, respectively, were generated by transfection using Lipofectamin LTX (Thermo Fisher Scientific, Waltham, MA) and selected with 200 μg/ml hygromycin. HEK293 cell clones were then transfected with a dual-luciferase reporter system (Cignal STAT3 Reporter, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Multiple repeats of a STAT3 binding site and basic promoter elements drive the expression of firefly luciferase. Renilla luciferase under the control of a CMV promoter serves as a control of transfection efficiency. Luciferase activity was determined in untreated cell and cells treated with either 50 μM S3I-201 (50 μM) or IL6 (10 ng/ml) after 24 h using the Dual-Glo® Luciferase Assay (Promega, Mannheim, Germany) and a Spark 10 M plate reader (Tecan Group, Maennedorf, Switzerland). Dual-luciferase results were calculated for each transfectant. The change in STAT3 activity was determined by comparing the normalized luciferase activities of the reporter in cells stably expressing mutant STAT3 compared to wildtype STAT3. Assays were performed in triplicates and repeated three times. One-way-ANOVA and Fisher LSD post-hoc significance were used for calculation of statistical significance.

2.9. Real-time PCR and gene expression arrays

Primary or EBV transformed cells derived from the patients or healthy controls were cultivated in the absence of serum for 16 h. Subsequently, cells were incubated in RPMI1640 supplemented with 2% FCS with or without IL21 (100 ng/ml), IL6 (25 ng/ml), S3I-201 (50 μM) or solvens (DMSO) for 24 h. Total RNA was then isolated using the RNeasy kit (Qiagen). cDNA was synthesized employing the QuantiTect Reverse Transcription kit. Real-time PCR was performed in triplicates and repeated at least two times using QuantiTect primers for *GAPDH, ACTIN*, *PPIA*, *SOCS3*, *BCL2*, *BCL-XL*, *BAX*, *BAK*, *FAS*, *FASLG* and the QuantiTect SYBR Green RT-PCR kit according to the manufacturer's recommendations (all from Qiagen). *GAPDH*, *PPIA* and *ACTIN* expression were used as an internal standard. Further gene expression analyses were performed using the RT^2 Profiler™ PCR Array Human Apoptosis according to the manufacturer's recommendations (Qiagen).

2.10. Immunophenotyping and ELISA

Immunophenotyping was carried out during routine clinical workout. In addition ALPS specific criteria were analyzed according to standard protocols employing the following antibodies: CD3 (clone UCHT1, BD Biosciences, Heidelberg, Germany), TCR αβ (clone T10B9.1A-31, BD Biosciences), CD4 (clone VIT4, Miltenyi), CD8 (clone BW135/80, Miltenyi), B220, HLA-DR, CD27, CD19, CD25 (all from BD Biosciences) and CD45R (Beckman Coulter, Krefeld, Germany). Cell surface expression of Fas and FasL was measured using anti-CD95 (clone DX2, BD Biosciences) and anti-CD178/FasL (clone NOK-1, Miltenyi) antibodies. FasL plasma levels were measured by ELISA (R&D-Systems) employing an Infinite M200 microplate reader equipped with Magellan software (Tecan).

2.11. Measurement of apoptosis

Activated primary T cells from the patients and healthy controls or EBV transformed B cells derived from the patient and healthy controls were plated in 96 well plates at a density of 10^6 cells/ml and treated with either recombinant SuperFasLigand (100 ng/ml, Enzo Life Sciences, Lörrach, Germany), 0.5 μM staurosporine (LC Laboratories, Woburn, MA), 100 ng/ml interleukin-21 (IL21) (Miltenyi), 1 nM-10 μM ABT-737 [8] (Selleckchem, Houston, TX), 10–50 μM S3I-201, mock-treated with solvens (DMSO) or left untreated. After 24 h cells were stained with Annexin V-FITC (BD Biosciences) and propidium iodide (PI, Sigma-Aldrich, Deisenhofen, Germany) and apoptosis was measured employing a FACSCalibur according to the manufacturer's suggestions (BD Biosciences) and analyzed using FlowJo software (v10, FlowJo LLC, Ashland, OR).

3. Results

3.1. Clinical characteristics of two unrelated individuals within a cohort of 30 patients with ALPS-like disease of unknown genetic cause

Patient 1 was born to consanguineous parents and suffered from early-onset hemolytic anemia, generalized lymphadenopathy and splenomegaly with subsequent development of autoimmune hypothyroidism and panhypogammaglobulinemia (Fig. 1A, and Table 1). Lymph node biopsy showed hyperplasia without evidence of infection or malignancy. Repeated immunophenotyping (Table 2) revealed normal absolute numbers of T, B, and natural killer cells, but increased double negative T cells (6–20%, reference range < 2.5%) and low regulatory T cells (0.4%, reference range 1–2%) in peripheral blood. She presented with increased numbers of naïve B cells (80% IgD + CD27-, reference range 39.5–76.3%) and B cells expressing only low levels of the CD21 co-receptor (21%, reference range 1.3–7.3%). Increased immunoglobulin M (IgM) serum levels with markedly reduced levels of IgG and IgA were observed, indicating Ig isotype class switch impairment. Steroid treatment caused improvement of the hemolytic anemia, but the patient continued to suffer from progressive lymphadenopathy and splenomegaly with episodes of immune-mediated thrombocytopenia. Short stature, recurrent *Herpes zoster* infections, glucose intolerance and elevated intraocular pressure were regarded as complications of steroid treatment. Mycophenolate mofetil was used to decrease the steroid dose resulting in the resolution of hemolysis and thrombocytopenia.

Patient 2 was born to non-consanguineous parents and presented with non-infectious, non-malignant lymphadenopathy, splenomegaly, thrombocytopenia, neutropenia, mild anemia and early-onset insulin dependent diabetes mellitus (Fig. 1B and Table 1). Double negative T cells were not elevated and immunoglobulin levels were normal (Table 2).

The presumptive diagnosis of ALPS was established for both patients.

3.2. Identification of two de novo heterozygous STAT3 germline mutations

Because neither germline nor somatic mutations in classical ALPS associated genes (*FAS, FASLG, CASP10*) were detected, whole exome sequencing was carried out to clarify the genetic cause of disease. A novel heterozygous germline mutation of *STAT3* (c.833G>A, p.R278H) was gained *de novo* in patient 1 (Supplementary Table 1) and confirmed by Sanger sequencing (Fig. 1C). A Combined Annotation-Dependent Depletion (CADD)-score of 23.9 indicated a deleterious mutation with a high likelihood of being pathogenic (Table 1). A somatic R278H mutation was recently detected in a diffuse large B cell lymphoma [23] and an R278C mutation in an endometrium derived carcinoma (COSMIC: Catalogue of Somatic Mutations in Cancer - cancer.sanger.ac.uk/cosmic database). The affected coiled-coil domain is associated with nuclear shuttling of STAT3 and binding of cytokine receptors [24]. In this domain, one other germline gain-of-function mutation (R152W) was reported previously [18]. The patient harboring the R152W mutation presented with ALPS-like symptoms: early-onset lymphadenopathy, hepatosplenomegaly, autoimmune hemolytic anemia and thrombocytopenia, and increased DNT cells (2.7–3.5%). In addition, insulin

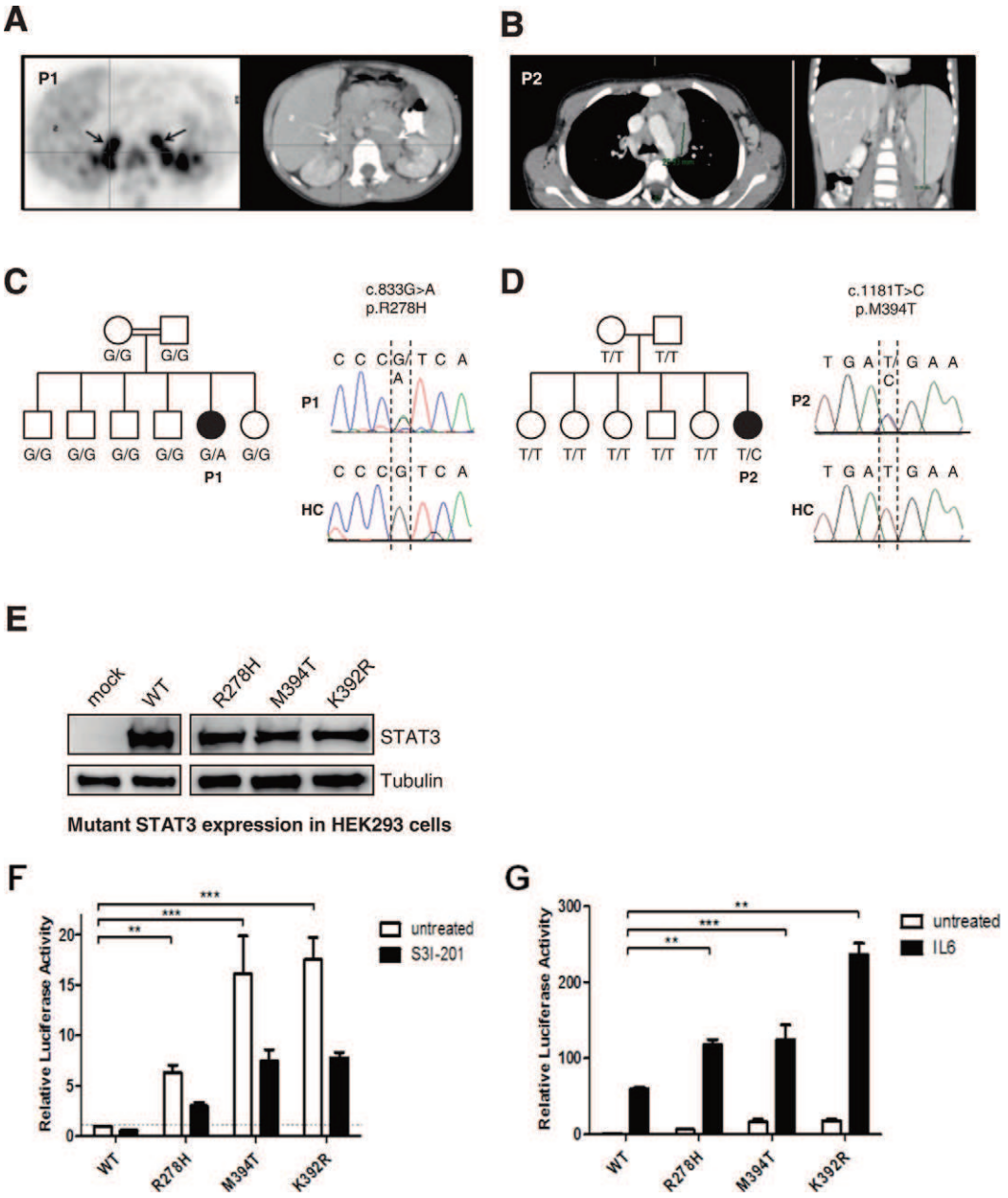


Fig. 1. Two *de novo STAT3* gain of function mutations were detected in ALPS-like patients. (A, B) Computer tomography of patient 1 (P1, A) and patient 2 (P2, B) demonstrates lymphadenopathy and splenomegaly. Coronal reformatted image of the abdomen reveals significant enlarged retroperitoneal lymph nodes of P1 (arrows). Lymphadenopathy and splenomegaly of P2 are indicated by green bars. (C, D) Family pedigrees of P1 (C), and P2 (D). P1 descended from consanguineous, P2 from non-consanguineous parents. P1 and P2 are the only carriers of the STAT3 mutations in their families (genotype indicated for each family member) and the only diseased family members (diseased state indicated by filled circle). Capillary sequencing of *STAT3* confirmed *de novo* mutations in patient P1 (C) and P2 (D). (E) The identified STAT3 mutations, a known detrimental STAT3 mutation (K392R) as well as STAT3 wildtype (WT) were cloned and expressed in HEK293 cells that lack intrinsic STAT3 expression. Expression was confirmed by western blot employing a STAT3 specific antibody and tubulin as a loading control. Mock transfected cells show absent baseline expression of STAT3. (F) STAT3 reporter assays in HEK293 cells stably expressing STAT3 wildtype and variants demonstrate hyperactivity of the patient derived mutations compared to wildtype STAT3. The activity of both mutant and wildtype STAT3 could be reduced by addition of the STAT3 inhibitor S3I-201. Mean values and standard deviations are shown ($^{**}p < 0.01$, $^{***}p < 0.001$). (G) STAT3 reporter assays in HEK293 cells stably expressing STAT3 variants performed as in (F) demonstrate that the activity of wildtype and mutant STAT3 is highly increased in response to IL6 (10 ng/ml, 24 h) ($^{**}p < 0.01$, $^{***}p < 0.001$).

dependent diabetes mellitus and recurrent *Herpes zoster* infections were observed.

In case of patient 2 whole exome sequencing revealed a previously reported *de novo STAT3* mutation that affected the DNA binding domain (c.1181T>C, p.M394T, Fig. 1D, Supplementary Table 2) [17]. The M394T mutation of *STAT3* was previously reported in one patient who presented with mild immune mediated thrombocytopenia, hypogammaglobulinemia, and disseminated mycobacterial disease in late adolescence. No autoimmunity or general lymphoproliferation, but increased numbers of DNT cells (5%) were observed [17]. In comparison, patient 2 presented in this study had a more severe phenotype, but did not suffer from mycobacterial disease or eczema.

3.3. The identified mutations confer elevated constitutive and inducible activity to the transcription factor STAT3

To analyze the impact of the identified mutations on the transcription factor function of STAT3, human embryonic kidney (HEK) 293 cells stably expressing STAT3 wildtype, the STAT3 variants R278H and M394T and STAT3 K392R as a positive control were generated and

Table 1
Clinical characteristics of the patients.

	Patient 1	Patient 2
STAT3 mutation	R278H *de novo*	M394T *de novo*
CADD score	23.9	25.4
Sex	Female	Female
Age at presentation (years)	3	2
Hematology	Chronic, generalized, non-infectious, non-malignant lymphadenopathy and splenomegaly, thrombocytopenia, intermittent leukopenia, anemia, hypogammaglobulinemia, DNT cells chronically elevated (6–20%)	Chronic, generalized, non-infectious, non-malignant lymphadenopathy and splenomegaly, thrombocytopenia and neutropenia. Mild anemia due to hemoglobin C trait.
Autoimmunity	Coombs positive hemolytic anemia, immune-mediated thrombocytopenia, auto-antibodies (ANA, anti-cardiolipin, thyroidites), RF, CRP and ESR chronically elevated	Insulin dependent diabetes, immune-mediated thrombocytopenia and neutropenia
Infection	Recurrent herpes zoster infection	No infections
Other clinical findings	Short stature, glucose intolerance, elevated intraocular pressure	Upper airway obstruction due to adenoid-tonsillar hypertrophy

(Abbreviations: ANA, antinuclear antibodies; CADD score, Combined Annotation-Dependent Depletion score; CRP, C-reactive protein; DNT cells, double negative T cells ($CD4^-CD8^-TCR\alpha/\beta^+$ T cells); ESR, erythrocyte sedimentation rate; RF, rheumatoid factor).

dual-luciferase reporter assays were carried out using promoter elements with multiple repeats of a STAT3 binding site that drive the expression of firefly luciferase. Luciferase activity was determined in untreated cells and cells treated with a cell permeable, small molecule inhibitor of STAT3 targeting the SH2 domain: S3I-201 (2-hydroxy-4-[[[[(4-methylphenyl)sulfonyl]oxy]acetyl]amino]-benzoic acid, NSC 74859). It was recently demonstrated that the inhibitor prevents phosphorylation, activation, dimerization, and DNA-binding of STAT3 [25]. Compared to wildtype STAT3, both patient derived mutants analyzed were transcriptionally increased active. M394T mutant *STAT3* has previously been shown to increase luciferase expression placed under a STAT3-specific promoter compared to wildtype controls [17]. Activity of both mutant and wildtype STAT3 could be diminished by application of the STAT3 inhibitor (Fig. 1G). After stimulation with IL6, the transcriptional activity of both wildtype and mutant STAT3 was highly increased, but mutant STAT3 still largely exceeded the activity of wildtype STAT3 (Fig. 1H). Taken together, both STAT3 mutations detected in ALPS like patients seem to confer constitutive activity to the transcription factor STAT3.

Phosphorylation at tyrosine residue 705 is required for dimerization and activation of STAT3 [15]. To determine whether the identified mutations have an impact on protein function we analyzed the phosphorylation state of STAT3 in patient cells. B cells of patient 1 and healthy controls were immortalized with *Epstein Barr virus* and lysates of these cells were used for western blot analyses.

pSTAT3 (Tyr705) levels were elevated in transformed R278H mutant cells compared to transformed control cells indicating that the R278H mutant is constitutively more active (Fig. 2A). To our knowledge

Table 2
Laboratory characteristics of patients P1 and P2.

	Patient 1	Patient 2	Ref. values
STAT3 mutation	R278H	M394T	
Total immunoglobulins			
IgM (mg/dL)	1460–2230	31–208	40–147
IgG (mg/dL)	<150–217	572–1470	680–1530
IgA (mg/dL)	<22.8	27–195	66–299
IgE (Iu/mL)	48	ND	<100
B cells (of gated lymphocytes)			
CD19+ (B cells) (%)	3–6	24	6–25
Kappa/Lambda ratio	1.0–1.6	1.2	1.2–2.9
IgD+/CD27- (naïve B cells) (% of CD19+)	80.9	ND	39.5–76.3
CD27+ (intermediate and memory B cells) (% of CD19+)	8.9	ND	7–20
CD21 low	21.7	ND	1.3–7.3
T cells (of gated lymphocytes)			
CD3+ (T cells) (%)	81–93	72%	55–84
CD4+/CD3+ (T helper) (%)	22–50.0	45%	31–60
CD8+/CD3+ (T suppressor) (%)	35–67	55%	13–41
Ratio CD4/CD8	0.5–1.4	0.8	1–3.6
DNT cells (% of CD3+/TCRα/β+)	6–20	<2.5%	<2.5%
B220+ (% of DNT)	10.3	ND	8–40%
CD25+ (% of CD3+)	9.1	ND	<18%
HLA-DR+ (activated T cells, % of CD3+)	33–38.4	ND	<10
CD4+/CD25+ (regulatory T cells, % of CD3)	<1%	ND	1–2
CD56+ (Natural killer cells) (%)	1–12	ND	5–27
CD25+/HLA-DR+ ratio	0.23	ND	2
Other parameters			
CRP	<0.32–10.6	<5–8.7	<1
WBC from CBC ($10^3/\mu L$)	3.7–5.2	2.2–4.3 (5–13)	4.3–10
Platelets ($10^3/\mu L$)	57–221	4–55	150–400
ANC ($10^3/\mu L$)	0.9–6	0.5–4	2–7
HgB (gr/dL)	5.5–15.5	9.4–12	10.5–15.0
sFASL (pg/ml plasma)	225	ND	<200

(Abbreviations: ANC, absolute neutrophil count; CBC, complete blood cells; CRP, C-reactive protein; HgB, hemoglobin; ND, not done; Ref. values, reference values; sFASL, soluble Fas ligand in plasma; WBC, white blood cells).

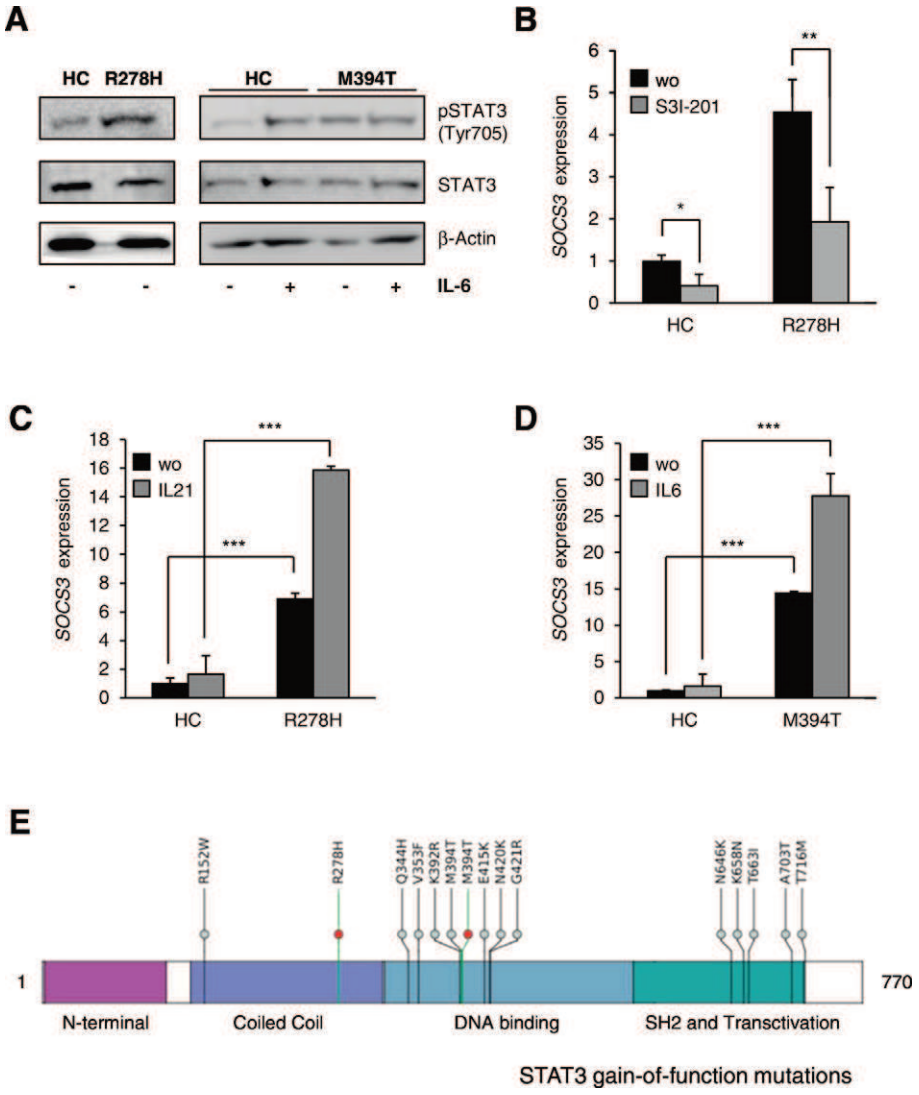
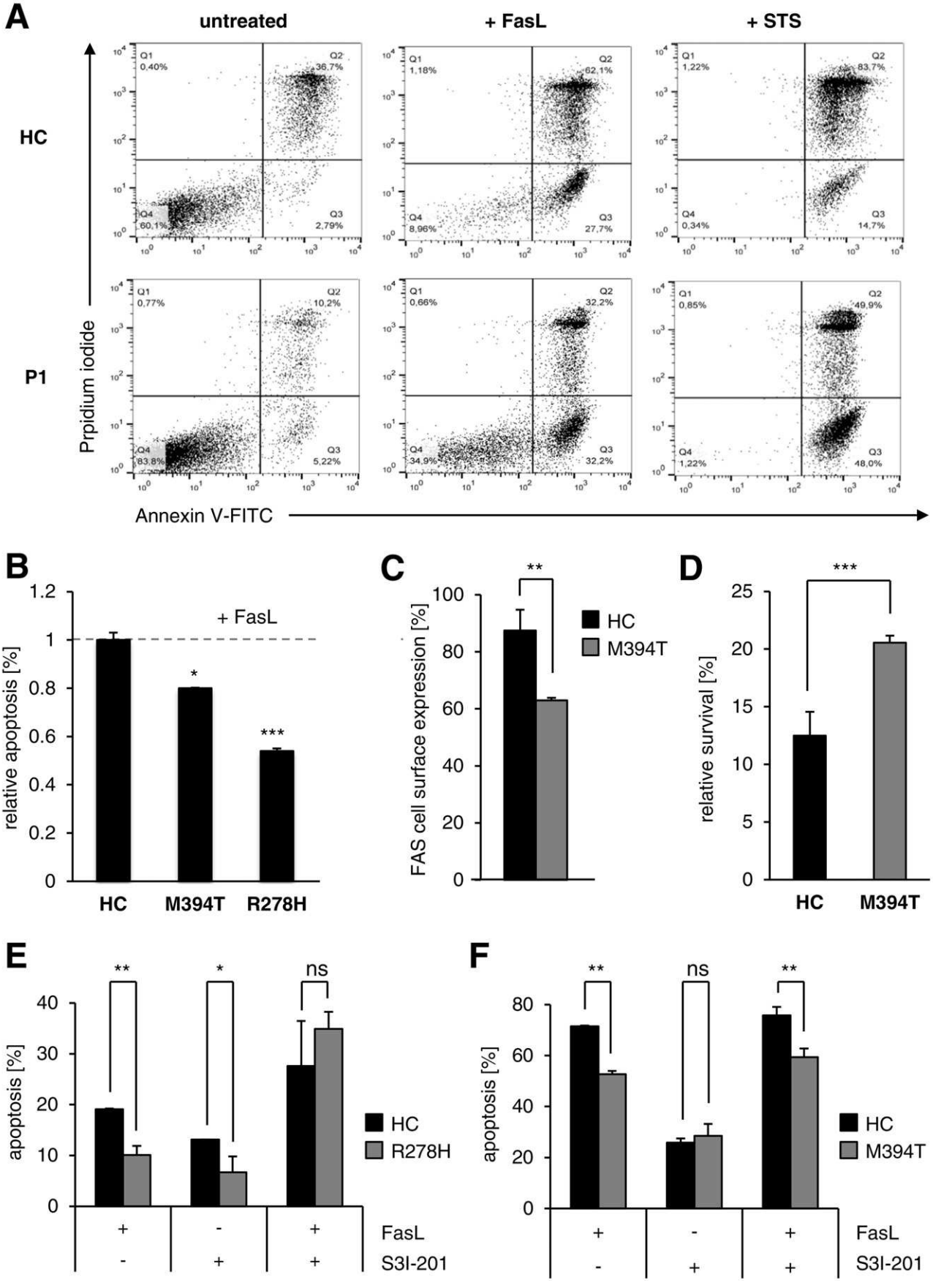


Fig. 2. Increased *STAT3* phosphorylation and expression of the downstream STAT3 target *SOCS3* further indicate gain of function mutations. (A) STAT3 phosphorylation at tyrosine 705 indicative of STAT3 activation is increased in cells derived from P1 and P2. Immunoblot analyses of total and phosphorylated (Tyr705) STAT3 protein employing specific antibodies is shown. β-Actin served as a loading control. *Left panel:* Lysates were prepared from EBV transformed B cells of a healthy control (HC) and patient P1 (harboring the R278H mutation). *Right panel:* Lysates were prepared from primary T cells of a healthy control and patient P2 (harboring the M394T mutation) after treatment with IL6 (25 ng/ml) for 24 h or untreated. (B) Expression level of the STAT3 target *SOCS3* is constitutively elevated in EBV transformed R278H B cells compared to EBV transformed healthy B cells (HC) and can be decreased by addition of the STAT3 inhibitor S3I-201 or (C) further increased by treatment with IL21 (100 ng/ml, 24 h). *SOCS3* mRNA expression was measured in triplicates by real-time PCR. Mean values and standard deviations are shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (D) *SOCS3* expression is upregulated in M394T mutant primary T cells from patient P2 compared to primary T cells from a healthy control (HC). *SOCS3* expression is further increased by treatment with IL6 (25 ng/ml, 24 h). Real-time PCR measurement was carried out as described in (B, C). (E) Schematic drawing of the STAT3 protein. The mutations identified in the patients (red dots) as well as other previously reported germline gain of function mutations are indicated (grey dots). The plot was generated using The Protein Painter application in the Pediatric Cancer Genome Project (http://explore.pediatriccancergenomeproject.org).

we demonstrate for the first time constitutive phosphorylation of STAT3 in primary cells of a patient: increased phosphorylation of STAT3 was observed under basal untreated conditions in lysates prepared from primary T cells of patient 2 (harboring the M394T mutation) compared to primary healthy control T cells. Treatment with interleukin-6 (IL6) led to increased STAT3 phosphorylation in healthy control cells indicative of activation, but did not further elevate the pSTAT3 (Tyr705) signal in cells derived from patient 2. Therefore, both detected *de novo STAT3* mutations seem to confer constitutive phosphorylation to the transcription factor STAT3.

To test this finding, we examined the expression of known STAT3 target genes. A main target of STAT3 is the suppressor of cytokine signaling 3 (*SOCS3*). We first isolated RNA from transformed B cells of patient 1 both under basal, untreated conditions and after activating STAT3 signaling by treatment with interleukin-21 (IL21) and carried out real-time PCR analyses. The results showed that *SOCS3* expression was highly increased in R278H mutant cells under basal and even more under stimulated conditions and could be decreased by addition of the STAT3 inhibitor S3I-201 (Fig. 2B, C). Similarly, *SOCS3* expression was upregulated in M394T mutant primary T cells derived from patient 2 compared to primary T cells from healthy controls under basal conditions and more enhanced after activation of STAT3 signaling by IL6 treatment (Fig. 2D). Germline gain-of-function mutations have been identified in all functional domains of STAT3 in humans. The majority of these mutations are situated in the DNA binding region and probably impact on the DNA-protein interaction (Fig. 2E) [26]. Other functions including stabilization of phosphorylation, dimerization, interaction with other proteins or nuclear shuttling may also be affected. The molecular basis underlying the gain of function of these mutant STAT3 proteins is currently unknown.


A
untreated
+ FasL
+ STS
HC
P1
Prpidium iodide
Annexin V-FITC
Q1 0,40%
Q2 36,7%
Q4 60,1%
Q3 2,79%
Q1 1,18%
Q2 62,1%
Q4 8,96%
Q3 27,7%
Q1 1,22%
Q2 83,7%
Q4 0,34%
Q3 14,7%
Q1 0,77%
Q2 10,2%
Q4 83,8%
Q3 5,22%
Q1 0,66%
Q2 32,2%
Q4 34,9%
Q3 32,2%
Q1 0,85%
Q2 49,9%
Q4 1,22%
Q3 48,0%
B
relative apoptosis [%]
+ FasL
HC
M394T
R278H
C
FAS cell surface expression [%]
HC
M394T
D
relative survival [%]
HC
M394T
E
apoptosis [%]
HC
R278H
FasL
S3I-201
F
apoptosis [%]
HC
M394T
FasL
S3I-201

3.4. Patient derived cells expressing hyperactive STAT3 are resistant against diverse apoptotic stimuli

Because clinical symptoms observed in the analyzed patients can be caused by defective lymphocyte cell death mediated by the Fas signaling pathway, we analyzed the expression of the Fas ligand and Fas receptor and tested the efficiency of Fas ligand mediated apoptosis in cells derived from the patients (Fig. 3A, B).

In patient 2 we detected a decrease of *FASLG* expression on RNA, but not on protein level employing flow cytometric detection of the Fas ligand protein at the cell surface (data not shown). Serum levels of soluble FASL were not significantly altered (Table 2). Using gene expression arrays, real-time PCR and flow cytometric analyses we detected a decrease in RNA and protein expression of the Fas receptor in M394T mutant primary T cells (Fig. 3C and Supplementary Fig. 1). These findings indicated a possible deficiency of Fas receptor mediated cell death. To test this hypothesis in primary cells derived from all three patients we used recombinant FASL to induce apoptosis *in vitro*. Primary T cells from the patients were indeed significantly less sensitive to recombinant FASL *in vitro* compared to controls confirming a deficiency of the Fas mediated apoptotic pathway (Fig. 3B). Interestingly, treatment of M394T mutant cells with STAT3 activating IL6 further protected cells from FasL induced apoptosis (Fig. 3D). To check whether the apoptosis defect was indeed associated with activated STAT3, we repeated the apoptosis tests using R278H mutant transformed B cells and pre-treated the cells with the specific, cell permeable STAT3 inhibitor S3I-201 [25] prior to addition of recombinant FASL. Indeed, the inhibition of STAT3 restored the apoptotic response of R278H mutant cells to FASL (Fig. 3E). This effect was not observed when primary M394T cells were similarly treated (Fig. 3F). This may be due to a lower effect of the S3I-201 inhibitor on M394T mutant STAT3 as indicated in reporter assays (Fig. 1G) or the higher sensitivity of cells expressing this mutant to FasL mediated apoptosis (Fig. 3B).

Because Fas mediated apoptosis is amplified in various cell types by the intrinsic mitochondrial apoptotic pathway, we also tested the functionality of this pathway in the patient cells using specific stimuli. Interestingly, transformed R278H mutant B cells were also clearly more resistant to intrinsic apoptotic stimuli like staurosporine or IL21 than comparable controls (Fig. 4A and Supplementary Fig. 2). This finding indicates that a combined defect of both the extrinsic and the intrinsic apoptotic pathway may cause the resistant phenotype of *STAT3* mutant patient cells.

3.5. Constitutive activation of the transcription factor STAT3 tips the balance of pro- and anti-apoptotic proteins of the BCL2 family to an apoptosis resistant state

Intrinsic apoptosis is tightly regulated by BCL2 proteins. To assess whether constitutively active STAT3 is associated with a disturbance of the balance of BCL2 proteins in patient cells, we carried out gene expression studies employing real-time PCR and expression arrays. In transformed R278H mutant B cells increased expression of resistance mediating anti-apoptotic BCL2 family members *BCL2* and *BCL-XL* (synonymously *BCL2L1*, Fig. 4B) was detected and confirmed on protein level employing immunoblot analyses (Fig. 4C). Expression of BCL2 family members that promote apoptosis, including *BAX* and *BAK1* was downregulated in R278H mutant cells compared to controls (Fig. 4B). Similarly, in primary M394T mutant T cells *BCL2* RNA levels were upregulated and *BAX* and *BAK1* downregulated (Fig. 4B). However, *BCL-XL* was not significantly differently expressed in M394T cells, indicating that different STAT3 mutations may elicit different disturbances of BCL2 protein family expression. Taken together our results indicate that the balance of pro- and anti-apoptotic BCL2 family proteins is tipped in favor of anti-apoptotic proteins rendering the patient derived cells more resistant to diverse apoptotic stimuli.

To test whether the alteration of the expression pattern is associated with STAT3 activation, we employed the STAT3 inhibitor S3I-201. IL-21 mediated apoptosis was associated with a downregulation of anti-apoptotic and upregulation of pro-apoptotic BCL2 family members in transformed B cells derived from healthy controls that was less pronounced in *STAT3* mutant cells (Fig. 4D). Similarly, expression of BCL2 was increased in M394T mutant cells upon stimulation with IL6 (Fig. 4E). Administration of the STAT3 inhibitor re-adjusted the expression of most *BCL2* family proteins in transformed R278H cells to levels comparable to normal cells (Fig. 4F). Pre-treatment with S3I-201 restored the apoptotic response of these cells to IL21 (Fig. 4G).

3.6. Cell death of lymphocytes expressing hyperactive STAT3 can be restored by the BCL2-inhibitor ABT-737

Our data suggested that activating *STAT3* mutations induce resistance to apoptosis by tipping the balance of BCL2 family members in favor of anti-apoptotic proteins. BH3 mimetic drugs such as the small molecule ABT-737 target the BH3-protein binding cleft of BCL2-like anti-apoptotic proteins and release sequestered BH3 proteins to induce apoptosis [8]. *Via* this mechanism ABT-737 efficiently kills cells that are dependent on *BCL2* expression for survival. Both R278H and M394T mutant cells overexpressed *BCL2*. We therefore tested whether the patient cells with constitutively active STAT3 respond to this drug. Both transformed R278H mutant cells (Fig. 5A) as well as primary M394T mutant T cells (Fig. 5B) were significantly more sensitive to ABT-737 compared to healthy control cells. In primary cells the effective dose was in the low nanomolar range and showed a considerably wide therapeutic window.

4. Discussion

Patients with hyperactive or constitutively active STAT3 share common clinical characteristics with ALPS patients, including lymphoproliferation and autoimmune cytopenias. ALPS patients frequently have increased IL10 plasma levels probably leading to hyperactive STAT3 signaling [27]. ALPS and STAT3 gain-of-function both predispose to lymphoid malignancies [9,10,14]. Interestingly, diffuse large B cell lymphoma may present with persistent STAT3 activation due either to elevated IL10 levels, STAT3 gain-of-function mutations or upstream events [28–30].

Fig. 3. *STAT3* mutant patient cells are less responsive to Fas ligand induced apoptosis. (A) Representative apoptosis measurement of primary T cells derived from patient P1 compared to a healthy control (sibling). Apoptosis was determined in untreated cells and cells treated for 24 h with either Fas ligand (FasL, 100 ng/ml) or staurosporine (STS, 0.5 μM) by flow cytometric measurement of phosphatidyl serine exposure employing Annexin V-FITC and propidium iodide. (B) Primary T cells derived from the patients induce apoptosis less efficiently than comparable healthy control cells. Primary T cells derived from peripheral blood of the patient and healthy controls were activated and expanded by PHA and IL2 treatment. Apoptosis was triggered by recombinant FAS ligand (100 ng/ml for 24 h) and measured by flow cytometry using Annexin V-FITC/propidium iodide staining. Depicted is the relative difference in the apoptosis rate of treated patient to corresponding treated wildtype cells (= 1). Mean values and standard deviations of a representative experiment of three, performed in duplicates, are shown ($^{*}p < 0.05$, $^{***}p < 0.001$). (C) Activated primary T cells of patient P2 (M394T mutant) express decreased levels of the death receptor FAS on their cell surface. FAS expression was measured by flow cytometry employing a FAS specific antibody. Mean values and standard deviations of a representative experiment of three are shown ($^{**}p < 0.01$). (D) IL6 protects M394T mutant patient cells from FasL mediated apoptosis. Apoptosis was measured as in (B). Cells were treated with FasL in the presence and absence of IL6 (25 ng/ml). Presented is the relative survival of FasL treated M394T primary T cells in the presence *versus* the absence of IL6. Mean values and standard deviations of a representative experiment of two are shown ($^{***}p < 0.001$). (E) Administration of the STAT3 inhibitor S3I-201 rescues the apoptotic response of R278H cells to FASL. Transformed R278H and healthy control cells were either mock-treated with solvent or treated for 24 h with S3I-201 (50 μM). Subsequently cells were treated with recombinant FASL (100 ng/ml) for further 24 h. Cells were harvested and apoptosis analyzed as described in (A). Mean values and standard deviations of a representative experiment of three are shown (ns, not significant, $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$). (F) S3I-201 does not further increase the apoptotic response of M394T primary T cells to FasL compared to healthy control cells. Representative experiment carried out as described in (E).

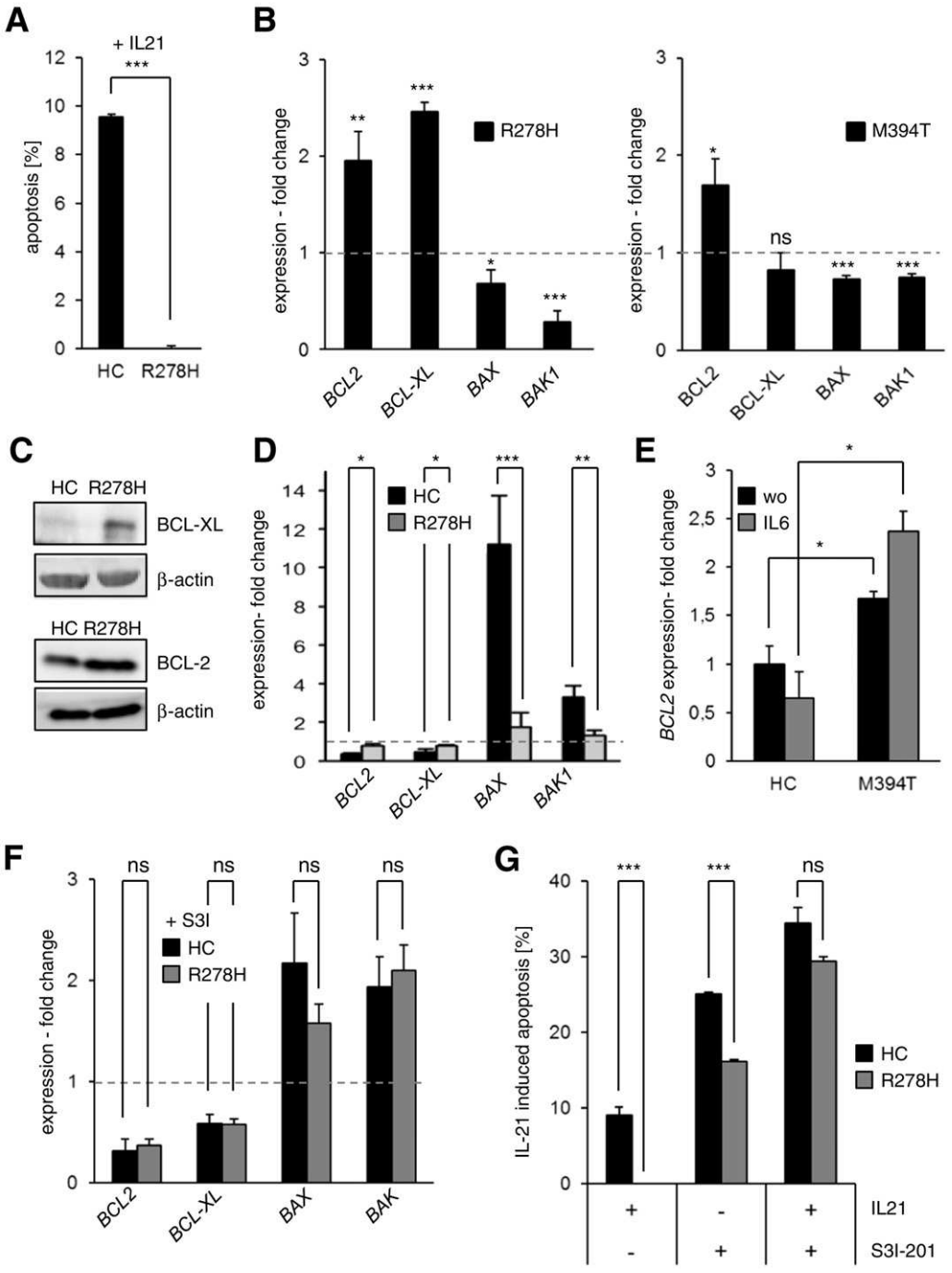


Fig. 4. *STAT3* gain of function mutations protect lymphocytes from apoptosis by dysregulation of the BCL-2 pathway. (A) EBV transformed R278H B cells are less susceptible to apoptosis induced by IL21 (100 ng/ml). Apoptosis was measured after 24 h of treatment as described in Fig. 3A. Mean values and standard deviations of a representative experiment of three are shown (***$p < 0.001$). (B) Constitutive activation of STAT3 shifts the balance of pro- and anti-apoptotic BCL-2 proteins in favor of apoptosis resistance. *Left panel*: Increased expression of anti-apoptotic *BCL2*, *BCL-XL*, and decreased expression of pro-apoptotic *BAX* and *BAK1* is demonstrated in EBV transformed R278H B cells compared to transformed control B cells by realtime-PCR. Experiments were carried out as described in (Fig. 2B, C) and the results of the R278H mutant cells are normalized relative to the healthy control (=1) (*$p < 0.5$, **$p < 0.01$, ***$p < 0.001$). *Right panel*: Similar measurements were carried out using M394T mutant and wildtype primary T cells. (C) Protein expression of anti-apoptotic BCL-2 proteins is elevated in transformed R278H cells compared to healthy control cells. Cell lysates were prepared and immunoblot analyses carried out as described in Fig. 1F. Specific antibodies against BCL2, BCL-XL, and β-Actin were employed. (D) Impaired induction of apoptosis by IL21 in transformed R278H cells was associated with a disturbed IL21 induced downregulation of *BCL2* and *BCL-XL* and upregulation of *BAX* and *BAK1* expression. Results of a representative real-time PCR experiment carried out three times in triplicates as described in Fig. 2B, C are shown. The results were normalized to the untreated results (=1) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (E) *BCL2* expression is downregulated in primary wildtype T cells in response to IL6, but further increased in primary M394T T cells. Representative real-time PCR experiment as described in (D). (F) Administration of the STAT3 inhibitor S3I-201 normalizes the expression of *BCL2* family genes in R278H cells treated with IL21. Transformed R278H and healthy control cells were either mock-treated with solvent or treated for 24 h with S3I-201 (50 μM). Subsequently, cells were treated with IL21 (100 ng/ml) for 24 h. Cells were harvested, RNA isolated and real-time PCR analyses carried out as described in Fig. 2B. Results were normalized to the results without added inhibitor. Mean values and standard deviations of a representative experiment of three are shown (ns, not significant). (G) Administration of the STAT3 inhibitor S3I-201 rescues the apoptotic response of R278H cells to IL21. Cells were treated as described in (F) and apoptosis was measured as described in Fig. 3A. Mean values and standard deviations of a representative experiment of three are shown.

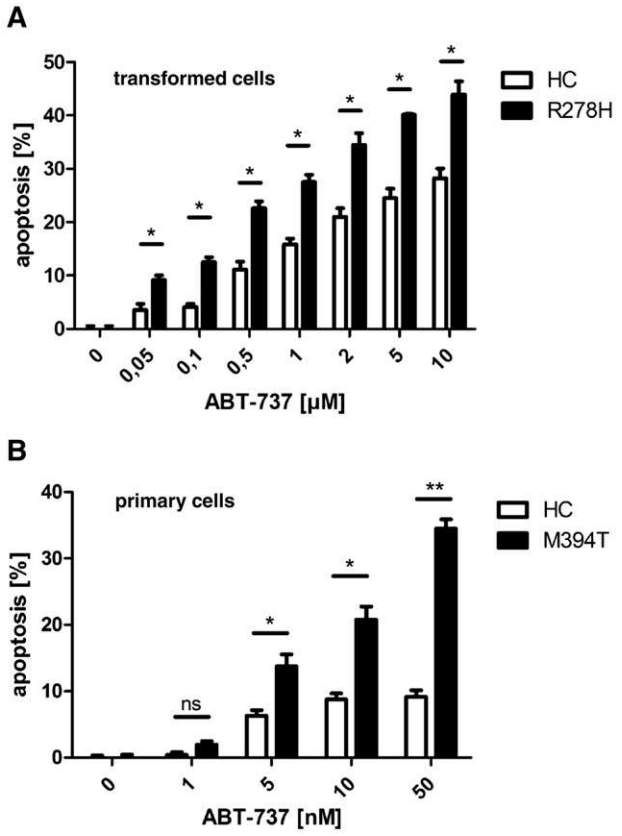


Fig. 5. The cell death defect caused by activated STAT3 can be compensated by a BH3 mimetic compound. (A) Transformed R278H mutant B cells are significantly more sensitive to ABT-737 than transformed wildtype B cells. Cells were either mock-treated with solvent or treated for 24 h with the indicated concentrations of ABT-737. Subsequently, apoptosis was determined as described in Fig. 3A. Mean values and standard deviations of a representative experiment of three are shown. Unpaired *t*-test was applied (*$p < 0.05$). (B) Primary M394T mutated T cells are significantly more sensitive to ABT-737 than comparable healthy primary T cells in a nanomolar range. Cells were treated as described in (A) with the indicated concentrations of ABT-737. Apoptosis was determined as in Fig. 3A. Mean values and standard deviations of a representative experiment of two are shown. Unpaired *t*-test was applied (ns, not significant, *$p < 0.05$, **$p < 0.01$).

The mechanistic cause of lymphoproliferation and perturbed apoptosis induction in STAT3 hyperactive syndrome has, however, not yet been characterized. ALPS patients consistently present with elevated numbers of DNT cells, but DNT cells may as well be increased in patients affected by other immune dysregulation syndromes [31] and in STAT3 hyperactive cases (5 of 13 [18], 1 of 3 [17] and 1 of 2 cases in the present study). We therefore assumed that hyperactive STAT3 could block lymphocyte apoptosis by deregulating apoptotic signaling pathways involved in the pathogenesis of ALPS or ALPS-like disease. An increase in lymphoproliferation caused by STAT3 hyperactivity was not observed in our study.

Deregulation of FAS was a likely cause for the ALPS-like symptoms of the patients in our study. Indeed, we could demonstrate that Fas expression was decreased in patient 2 and Fas mediated apoptosis was deficient in primary T cells derived from both analyzed patients. This effect was probably closely linked to STAT3 activity in the cells of our patients, because inhibition of STAT3 in R278H cells, using the small molecule STAT3 inhibitor S3I-201, led to reconstitution of FAS mediated apoptosis.

It has previously been demonstrated that STAT3 can repress *FAS* transcription [32]. The *FAS* gene promoter contains binding sites for STAT3 (−460 to −240 bp) and direct binding of STAT3 in complex with c-Jun, has been confirmed using EMSA and ChIP assays. It is probable, that distinct mutations may affect STAT3 function in different ways. In this line Milner et al. [18] observed that mutations in the DNA binding domain caused constitutive activation of STAT3, whereas a mutation (p.K658N) in the dimerization domain only conferred hypersensitivity to interleukins. Depending on the effect of the respective *STAT3* mutation on *e.g.* target recognition or interaction with co-factors, distinct mutations of *STAT3* may achieve different levels of suppression of FAS expression and FAS mediated apoptosis. In their cohort of 15 patients with hyperactive STAT3 and without common ALPS mutations, Milner et al. [18] tested susceptibility to Fas mediated apoptosis in activated primary cells expressing five (of nine) different *STAT3* mutations (p.T663I, p.R152W, p.V353F, p.N420 K, p.A703T). Strikingly, p.A703T mutant cells showed no significant cell death induction at all after stimulation with an agonistic Fas antibody (CH11) compared to controls. A similar effect was reportedly not observed for the other mutations, but the data was not included. Our data showed a partial defect in Fas mediated apoptosis, therefore the difference between our studies may be due to different cut offs. Treatment of the patients at the time of sample drawing might also have adversely affected the assay. Nevertheless, taken together, these findings strongly indicated a possible impact of disturbed Fas receptor signaling on the clinical symptom of lymphoproliferation in at least some patients with STAT3 hyperactivity, but also suggested that other additional mechanisms may play a role.

Our results provided evidence for a dysregulated BCL2 pathway as an important mechanism for lymphoproliferation and autoimmunity in patients with *STAT3* gain-of-function mutations. The anti-apoptotic state of the cells seemed to be closely correlated with an overexpression of anti-apoptotic BCL2 proteins. Interestingly, the main anti-apoptotic BCL2 proteins (BCL-2, BCL-XL and MCL-1) are all direct targets and positively regulated by the STAT3 transcription factor [33–36]. STAT3 is known to maintain T cell homeostasis by regulating the expression of pro-apoptotic and anti-apoptotic factors such as BCL2, BCL-XL, BAX, BAK1 and BIM. Recently, it could be demonstrated that the number of thymocytes and T cells in the spleen and lymph nodes of *STAT3*-deficient mice was significantly decreased due to a higher susceptibility to apoptosis [37]. Interestingly, expression of BCL2 and BCL-XL was significantly reduced in *STAT3*-deficient thymocytes and T cells. Moreover, it could be shown that STAT3 enhances survival of activated T cells by upregulating the expression of BCL2 while downregulating the expression of FAS ligand and BAD [38]. STAT3 seems to function as an anti-apoptotic factor, especially in numerous malignancies, where STAT3 is constitutively active/phosphorylated [39,40].

It was recently demonstrated that the BCL2 pathway was deregulated in DNT cells of 12 ALPS patients and that the release of overexpressed BIM by the BCL2 inhibitor ABT-737 rendered the cells susceptible to apoptosis [27]. Similarly, in our study a deregulation of BCL2 family proteins sensitized the cells to this inhibitor. Our data therefore implicate BH3 mimetics as potential therapeutics for patients with *STAT3* gain-of-function mutations. ABT-737 was effective and well tolerated in mice models of autoimmune disease [41]. Side effects were restricted to dose dependent lymphopenia and thrombocytopenia. The BCL2 specific, synthetic derivative ABT-199, currently in clinical development (phase II and III trials), is an alternative drug devoid of toxicity against platelets and targeted delivery could further improve treatment specificity. It would further be interesting to see whether BH3 mimetics would also address the tumor predisposition in *STAT3* gain-of-function mutated patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2017.05.021.

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Specific antibody deficiency and autoinflammatory disease extend the clinical and immunological spectrum of heterozygous *NFKB1* loss-of-function mutations in humans

The nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB1) is a master regulator of immune and inflammatory responses.[1,2] NF-κB1 belongs to the NF-κB/Rel family of transcription factors that consists of five members in humans: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA, c-Rel, and RelB. The p105 and p100 precursors are proteolytically processed by the proteasome to generate the shorter p50 and p52 isoforms. Homo- and heterodimers are formed by p50, p52 and the Rel proteins. Unstimulated, these dimeric complexes are sequestered in the cytoplasm by inhibitory IκB proteins in an inactive state. Upon stimulation, the phosphorylation and subsequent degradation of IκB proteins is rapidly triggered, releasing the NF-κB/Rel complexes to enter the nucleus where they bind to DNA at κB sites and activate or repress the expression of their target genes.[3]

Recently, heterozygous mutations affecting the *NFKB1* gene were identified in three human families. Haploinsufficiency of NF-κB1 was causative for combined variable immunodeficiency (CVID) characterized by recurrent infections due to immunoglobulin deficiency (pan-IgG, IgA and/or IgM).[4]

We report here on two pediatric patients from unrelated families with two novel *NFKB1* gene mutations identified by whole-exome sequencing (Figure 1A,B). Both patients had early onset of disease during their teenage years and presented in addition to hypogammaglobulinemia or selective IgA deficiency with a striking lack of specific antibodies (clinical characteristics are summarized in Table 1 and *Online Supplementary Table S1*).

Patient 1 is a now 26-year old female who first presented with recurrent autoimmune hemolytic anemia at the age of 14. Hypogammaglobulinemia (IgG2 subclass deficiency), deficient production of specific antibodies, decreased class-switched and memory B cells, naïve CD4-positive and regulatory T cells, increased activated and double-negative T cells (DNT cells, $CD4^{-}CD8^{-}$ $TCR\alpha/\beta^{+}$), autoimmune phenomena (hemolytic anemia, thrombocytopenia and leukopenia), lymphadenopathy, and hepatosplenomegaly were observed. She developed chronic lung disease with bronchiectasis, frequent respiratory tract infections and pneumonia. Infections with viral, bacterial and fungal pathogens were frequent. She suffered from intractable abdominal pain and bloody diarrhea without evidence of infection. After a liver biopsy she developed pancolitis with subsequent sepsis and multi-organ failure and was successfully resuscitated. The patient is being treated with intravenous immunoglobulin. Steroids were intermittently given to reduce pulmonary infiltrates with partial response. Mycophenolate mofetil stabilized blood counts, but pulmonary symptoms and infections remained.

To identify the genetic cause of disease whole exome sequencing was performed for the patient and her family (*Online Supplementary Methods*, *Online Supplementary Table S2*). A heterozygous *de novo NFKB1* frameshift mutation was detected (Figure 1A). The *NFKB1* gene encodes two proteins: p50 and its precursor p105. The mutation led to early protein truncation (I47YfsX2) and decreased expression of both protein isoforms (Figure 1B,C). RNA expression was slightly reduced.

Patient 2 is a 19-year old female from a consanguineous family (Figure 1A), who presented at the age of 11 with recurrent respiratory infections leading to chronic lung disease with bronchiectasis. She suffered from lymphadenopathy, splenomegaly, recurrent autoimmune hemolytic anemia and diarrhea. Whole exome sequencing revealed an inherited heterozygous nonsense *NFKB1* mutation (R157X) leading to early protein truncation and decreased p50/p105 expression (Figure 1B,D). *NFKB1* mRNA levels were low suggesting that the mutation may lead to mRNA instability and nonsense-mediated decay. Sequencing of reverse transcribed mRNA (Figure 1C,D, lower panels) indicated the absence of mutated mRNA in cells of patient 2, but not patient 1. Patient 2 had a selective IgA deficiency; her IgG and IgM levels were within the normal ranges. Similar to patient 1, she lacked specific antibodies including isohemagglutinins. Immunophenotyping demonstrated normal absolute numbers of T, B and natural killer cells, but decreased numbers of naïve T cells and increased levels of activated, DNT and $TCR\gamma/\delta^{+}$ T cells. Her splenomegaly and bronchiectasis are shown in Figure 1E. The father and two siblings carry the same mutation, so far without clinical signs except low IgM, IgG1 and IgA levels (*Online Supplementary Table S3*). Elevated levels of IgG2, IgG3 or IgG4 may compensate for lack of IgG1 in these mutation carriers.

To analyze the impact of the mutations on NF-κB mediated signaling, we tested the response to stimuli known to trigger the canonical NF-κB pathway (Figure 2A). We treated Epstein Barr virus-transformed B cells of patient 2 and healthy controls with lipopolysaccharide (LPS), tumor necrosis factor α or interleukin 1β and analyzed expression of *NFKB1* (itself a target of NF-κB-mediated regulation). *NFKB1* expression was upregulated in control, but not in R157X-mutant cells. The lack of a measurable *NFKB1* transcriptional response in the heterozygous cells suggested a more profound effect of the mutation than mere p50/p105 dose reduction would explain. Upregulation of other target genes, such as *CFLAR*, upon activation was deficient in R157X-mutant cells (Figure 2B). To analyze the alteration of NF-κB-regulated gene expression in more detail we used predesigned real-time polymerase chain reaction assays that include 84 NF-κB targets (*Online Supplementary Figure S1*). The basal expression pattern of target genes was largely different in wild-type compared to R157X-mutant cells. LPS stimulation demonstrated stark differences in inducible NF-κB target gene expression. Of the NF-κB genes, *RELA* and *RELB* were similarly expressed in mutant and control cells (at baseline and stimulated), whereas *NFKB1*, *NFKB2* and *cREL* were expressed less in mutant cells and only *cREL* showed inducible expression upon LPS treatment (*Online Supplementary Figure S2*). Only 12 of 79 evaluable genes showed a comparable expression pattern in wild-type and *NFKB1* mutant cells indicating compensatory regulation or independence of p50/p105 in the presence of other NF-κB proteins. When we compared primary T cells of patient 1 to those of two healthy controls (Figure 2C) baseline expression of all NF-κB genes was reduced and 40 more known targets were differentially regulated. Both patients demonstrated disturbed expression and regulation of the death receptor FAS. Instead of downregulating FAS expression during infection to allow for effective immune response, LPS induced FAS upregulation (*Online Supplementary Figure S1*). Accordingly, *in vitro* stimulation of primary T cells with recombinant Fas ligand resulted in significantly decreased cell death in the presence of LPS or tumor necrosis factor α in control cells, but not in cells derived

from both patients (Figure 2D). Both patients presented with clinical features similar to those of the Fas signaling pathway associated autoimmune lymphoproliferative syndrome (increased DNT cells, hepatosplenomegaly, lymphadenopathy, autoimmune symptoms, low regulatory T cells), but Fas ligand-induced apoptosis was not deficient, autoimmune lymphoproliferative syndrome-associated gene defects were absent and infections were

Table 1. Clinical and laboratory characteristics of two patients with novel *NFKB1* mutations.

	Patient 1	Patient 2	Normal value
Mutation	p.Ile47TyrfsX2 (exon 4)	p.Arg157X (exon 7)	
Sex	Female	Female	
Born	1990	1997	
Age	14 years (at presentation)	11 years (at presentation)	
Hematology	Hepatosplenomegaly, lymphadenopathy, panhypogammaglobulinemia, specific antibody deficiency, increased DNT cells, decreased B cells, naïve and regulatory T cells, SIRS after liver biopsy	Splenomegaly, selective IgA deficiency, specific antibody deficiency, increased DNT and TCR γ/δ+ T cells, decreased naïve T cells	
Autoimmunity	AIHA, ITP, pancytopenia, autoantibodies	AIHA, ITP, leukopenia	
Infection, inflammation	Recurrent pneumonias, URTI, fever, chronic otitis, mastoiditis, sinusitis, vulvovaginitis	Recurrent pneumonias, URTI	
Pathogens	Recurrent viral, bacterial and candida infections (chronic EBV, recurrent Herpes zoster; vaginal: HPV83, HSV, Gardnerella and group B Strep., *Citrobacter braakii*, *Candida lusitaniae*; oral: HSV, RSV; blood: EBV, HHV6; ear: *Staph. aureus*, group A Strep.; colon: Adenovirus)	*Streptococcus pneumonia*, *Haemophilus influenza*, Salmonella infections	
Gut disease	Pancolitis, recurrent idiopathic diarrhea, abdominal pain, bloody stools, ascites	Recurrent diarrhea	
Other clinical findings	Chronic inflammatory lung disease, organ infiltration (liver, lung, spleen, kidney), nephromegaly, impaired kidney function, intermittent proteinuria, latent hypothyreosis, multiple ovarian cysts, lack of calcium and vitamin D, recurrent headaches with vertigo, numbness and paresis of left arm and hand, generalized mucositis, recurrent aphthae and painful ulcers of mouth, esophagus and genitalia	Chronic lung disease, bronchiectasis, clubbing	
Treatments	IVIG, steroids, mycophenolate mofetil, antibiotics, antiviral medication, calcium, vitamin D	IVIG, steroids	
Surgery	Cholecystectomy, colectomy, mastoidectomy, ulcer excision, myringotomy and tympanostomy tube insertion		
Total immunoglobulins:			
Age at examination	22	19	
IgM (mg/dL)	24	362	34-250
IgG (mg/dL)	485	1091	680-1530
IgA (mg/dL)	<6.2	<5	66-300
Specific antibodies:			
Isohemagglutinins	Anti-A1 1:4 Anti-A2 1:4 Anti-B 1:2 (blood group O Rh)	absent	
Anti-diphtheria toxoid	< 0.1 IU/mL		low protection: >0.01-0.1; protection: >0.1
Anti-tetanus toxoid	< 0.1 IU/mL		low protection: >0.01-1.0; protection: >1.0
Others		Lack of antibodies against measles, mumps, rubella, hepatitis A and B	

AIHA: autoimmune hemolytic anemia; DNT: double negative T cells; EBV: Epstein Barr virus; HHV: human herpes virus; HPV: human papilloma virus; HSV: herpes simplex virus; IgA: immunoglobulin A; ITP: idiopathic thrombocytopenia; IVIG: intravenous immunoglobulin; RSV: respiratory-syncytial-virus; SIRS: systemic inflammatory response after surgery; Staph.: Staphylococcus; Strep.: Streptococcus; TCR: T cell receptor; URTI: upper respiratory tract infection.

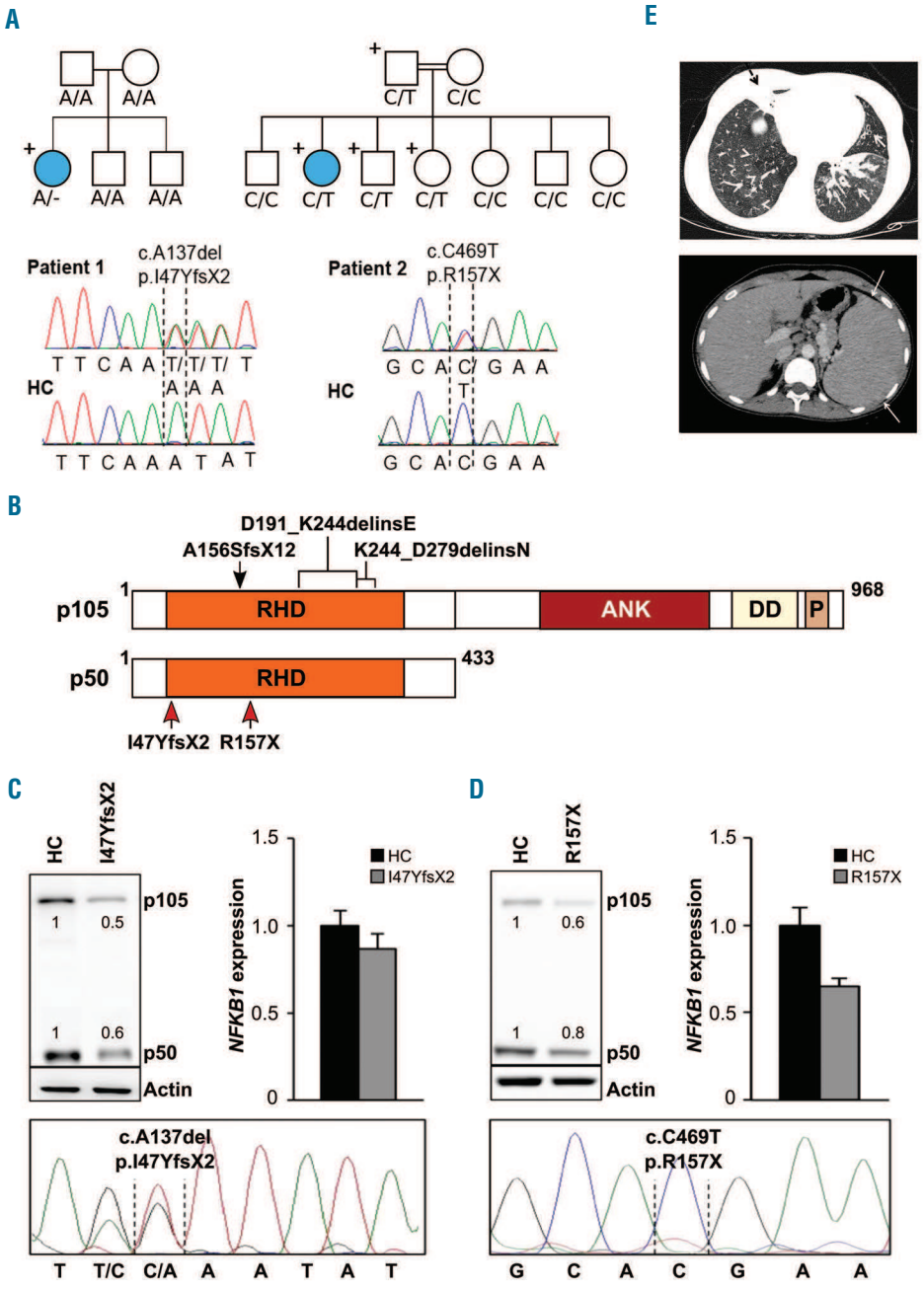

Figure 1. Two novel heterozygous *NFKB1* mutations detected in two families decrease the protein levels of p105 and p50. (A) Upper left: Whole exome sequencing identified a heterozygous *NFKB1* mutation (A/-) in patient 1. The patient is the only carrier of the mutation in the family pedigree (indicated by "+") and the only diseased family member (indicated by a filled circle). Lower left: Capillary sequencing using genomic DNA confirmed an *NFKB1* frameshift mutation (c.A137del, p.I47YfsX2) in patient 1. Representative chromatograms of patient 1 and a healthy control (HC) are shown. Upper right: Patient 2 descended from consanguineous parents and harbors an inherited heterozygous *NFKB1* mutation (C/T). The patient is the only diseased family member. The father and two siblings carry the same mutation but are not affected. Sanger sequencing of *NFKB1* confirmed the heterozygous missense mutation (c.C469T, p.R157X) in patient 2. Representative chromatograms are shown. (B) Schematic drawing of the proteins p105 and p50 and their domains which are both encoded by the *NFKB1* gene. The mutations in the Rel homology domain (RHD) identified in the two patients (red arrows) lead to early truncation of both proteins. Previously reported heterozygous germline mutations associated with CVID are indicated on top (black arrow and brackets). ANK, ankyrin repeats; DD, death domain; P, PEST domain enriched for proline (P), glutamic acid (E), serine (S), and threonine (T) residues. (C, D) Expression of p105 and p50 proteins is decreased in the affected patients. (C) Primary T cells of patient 1 and healthy controls were activated by phytohemagglutinin in the presence of interleukin-2. Protein and RNA extracts were prepared. Western blot analysis was carried out employing a specific p105/p50 antibody using β-actin as a loading control (left panel). *NFKB1* mRNA expression was measured by real-time polymerase chain reaction (right panel). The fold-change in cells of the patient compared to a representative healthy control is shown, GAPDH and β-actin expression were used as internal standards. Mean values of representative experiments performed in triplicates and corresponding SDs are shown. Sanger sequencing using reverse transcribed mRNA of the patient demonstrates the presence of mutated *NFKB1* transcripts (lower panel). (D) Epstein-Barr virus-transformed B cells of patient 2 were used for protein and RNA extraction. Analysis of NFKB1 protein and RNA expression was carried out as described in (C). Capillary sequencing of cDNA from patient 2 failed to detect the *NFKB1* mutation indicating that the mutation leads to mRNA instability (lower panel). (E) Upper panel: axial high resolution chest computer tomography image of patient 2 at the level of lung bases demonstrating multiple areas of bronchiectasis (white arrows) and consolidation with an atelectatic component surrounding bronchiectases in the right middle lobe (black dashed arrow). Mosaic pattern of perfusion of the lung parenchyma is noted, with multiple areas of low attenuation in the right low lobe (arrowheads). Lower panel: axial computer tomography image of patient 2 at the level of the upper abdomen demonstrating the enlarged spleen.

too frequent.

The gastrointestinal symptoms of patient 1 were reminiscent of ulcerative colitis, which is acknowledged to be a systemic autoimmune disease of unknown genetic cause. A candidate gene is *NFKB1*.[5] Susceptibility to inflammatory bowel disease can be caused by mutant *NOD2*, which fails to activate NF-κB in response to bacterial infection.[6] An *NFKB1* promoter polymorphism (-94delATTG) leading to lower LPS-induced gene expression was described in inflammatory bowel disease.[7]

A characteristic of both presented patients was inadequate development of specific antibodies. The genetic cause of specific antibody deficiency is not known, but heterozygous deletion of IkBα led to functional NF-κB haploinsufficiency and lack of specific antibody responses after immunization.[8] Reduced p50 expression may specifically play a role. Mice with targeted p50 disruption were deficient in producing specific antibodies in response to vaccination,[9] whereas mice lacking only the C terminal half of the p105 precursor, but expressing the N terminal p50 protein (p105$^{-/-}$), were not.[10]

Immunoglobulin class switching is known to be regulated by NF-κB. Mutations in the NF-κB essential modulator (NEMO) cause a class switch defective hyper-IgM syndrome in humans.[12] Decreases in IgG, IgA, IgE, but not IgM were observed in p50 knockout mice.[9-11] NF-κB regulates the expression of activation-induced cytidine deaminase (AID encoded by the *AICDA* gene), an enzyme required for DNA cleavage during class switch recombination. *AICDA*-knockout mice and patients with *AICDA* mutations are both class switch deficient. Thus, low levels of NF-κB may impair AID-mediated class switch recombination.[13]

Loss of p105 in the patients may play a role in the pathogenesis of infections and inflammatory disease, lymphadenopathy and splenomegaly that were also

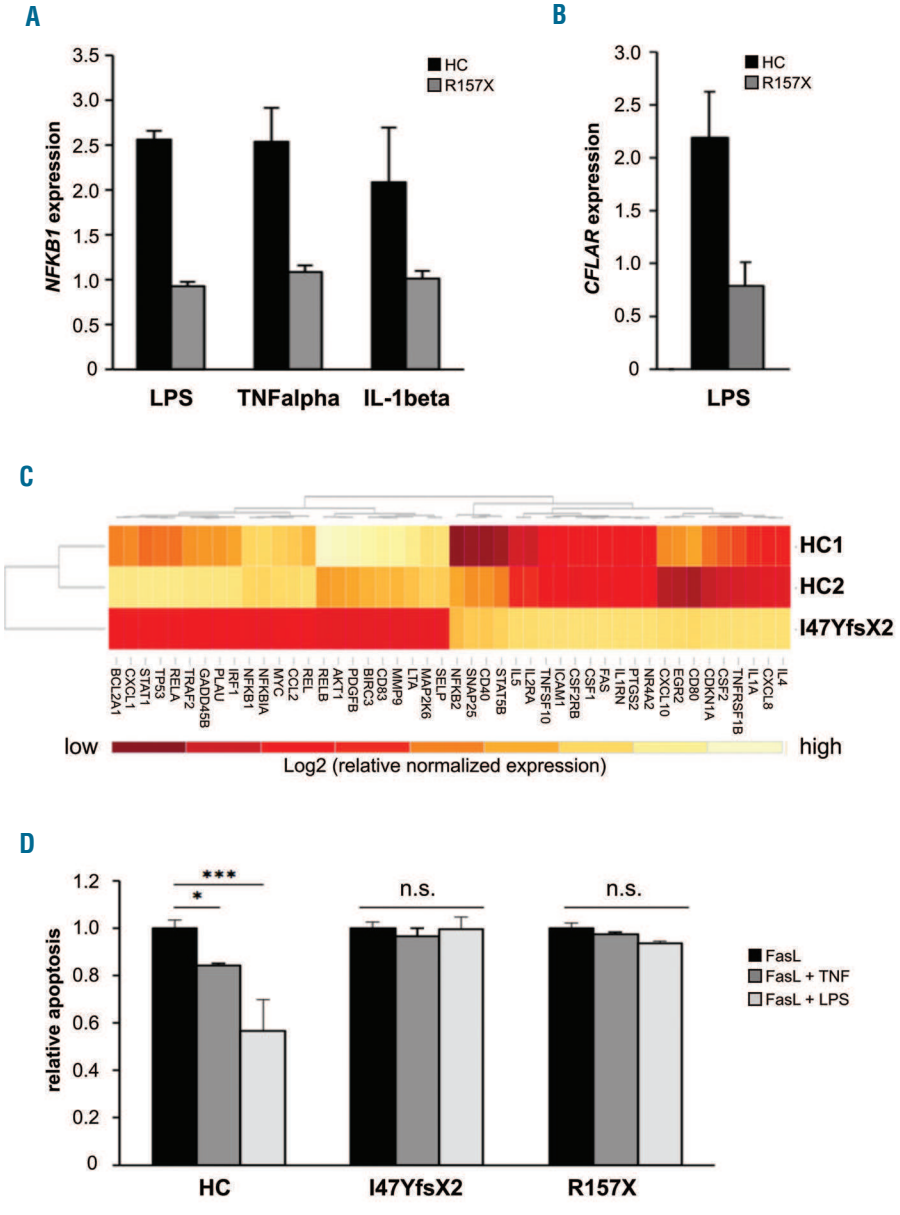

Figure 2. NF-κB-mediated signaling is affected in the patients. (A) Upregulation of *NFKB1* mRNA expression in response to NF-κB activating stimuli is deficient in *NFKB1*-mutated cells. Epstein-Barr virus (EBV)-immortalized B cells of patient 2 and of a representative healthy control (HC) were treated for 16 h with LPS (5 μg/mL), tumor necrosis factor α (TNFα) (50 ng/mL) or IL-1β (10 ng/mL). Fold change of *NFKB1* mRNA expression in untreated compared to treated samples was determined by real-time polymerase chain reaction (PCR). The relative expression was normalized to the respective untreated controls (=1). GAPDH and β-actin expression were used as internal standards. Mean values of representative experiments performed in triplicate and corresponding standard deviations are shown. (B) Expression of the NF-κB target gene *CFLAR* (synonymous for cFLIP) is not induced by LPS in the *NFKB1*-mutated patients' cells. EBV-immortalized B cells of patient 2 and healthy control cells were treated for 16 h with LPS (5 μg/mL). Fold change of mRNA expression of *CFLAR* was determined by real-time PCR as described in Figure 1C. (C) Differential expression of NF-κB target genes in *NFKB1*-mutated primary T cells of patient 1 and two healthy wild-type controls. Baseline expression of a panel of NF-κB target genes was analyzed by real-time PCR using predesigned arrays (NF-κB signaling targets RT^2 Profiler PCR arrays, Qiagen, Hilden, Germany). Mean values of two independent assays are shown. High gene expression is indicated in light color, low gene expression in dark red. The analysis was supervised and results are shown scaled. (D) LPS does not protect *NFKB1* mutant primary T cells from apoptosis. Primary T cells of patients 1 and 2 and healthy controls were activated by phytohemagglutinin and IL2. Cells were treated with 100 ng/mL recombinant Fas ligand to induce apoptosis in the presence or absence of 100 ng/mL LPS or 10 ng/mL TNFα. Apoptosis was determined by flow cytometric measurement of phosphatidylserine exposure indicated by binding of annexin V-FITC. Dead cells were detected with propidium iodide. Significance was tested using two-way ANOVA (*P<0,05; ***P<0,001).

observed in the mouse model.[10] p105 knockout led to constitutive activation of p50 homodimer repressors/activators indicating that low expression of *NFKB1* may result in a severe dysregulation of the NF-κB network.

Similar to other gene defects with an impact on immune system control, *NFKB1* mutations lack complete penetrance.[14] In three pedigrees with 20 individuals harboring mutant *NFKB1* alleles, the age at onset of disease varied from 2-64 years. Two gene carriers were completely healthy, while others had mild phenotypes with transient hypogammaglobulinemia or mild infections.[4] Gender may be a bias, because most clinically affected individuals were females (17 of 22 cases). In our patients, we did not detect mutations in possible modifier genes on the X chromosome (e.g. *FOXP3*, *TLR7*). Taking into account that our and most of the reported cases (18 of 20) were affected during or after puberty we could speculate that X-linked hormonal differences may contribute to the higher penetrance of the disease in females. In this line, one of our patients showed reduced numbers of regulatory T cells. The number of these cells can also be affected by hormonal fluctuations.[15]

Our results broaden the phenotypic spectrum of *NFKB1*-associated disease and have novel implications for the diagnosis and treatment of patients harboring *NFKB1* mutations and the therapeutic application of NF-κB directed drugs.

Cyrill Schipp,[1] Schafiq Nabhani,[1*] Kirsten Bienemann,[1] Natalia Simanovsky,[2] Shlomit Kfir-Erenfeld,[3] Nathalie Assayag-Asherie,[3] Prasad T. Oommen,[1] Shoshana Revel-Vilk,[4] Andrea Hönscheid,[1] Michael Gombert,[1] Sebastian Ginzel,[1,5] Daniel Schäfer,[1] Hans-Jürgen Laws,[1] Eitan Yefenof,[3] Bernhard Fleckenstein,[6] Arndt Borkhardt,[1] Polina Stepensky,[*4] and Ute Fischer[*1]*

**CS and SN as well as PS and UF contributed equally to this work.*

[1]*Department of Pediatric Oncology, Hematology and Clinical Immunology, University Children's Hospital, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany;* [2]*Department of Radiology, Hadassah Hebrew University Medical Center, Jerusalem, Israel;* [3]*The Lautenberg Research Center, Hebrew University-Hadassah Medical School, Jerusalem, Israel;* [4]*Pediatric Hematology Oncology and Bone Marrow Transplantation Department, Hadassah Hebrew University Medical Center, Jerusalem, Israel;* [5]*Department of Computer Science, Bonn-Rhine-Sieg University of Applied Sciences, Sanket Augustin, Germany; and* [6]*Department of Clinical and Molecular Virology, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany*

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Correspondence: ute.fischer@med.uni-duesseldorf.de

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