

# **Common variable immunodeficiency, impaired neurological development and reduced numbers of T regulatory cells in a 10-year-old boy with a STAT1 gain-of-function mutation**

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## Abstract

Recently, gain-of-function (GOF) mutations in the gene encoding signal transducer and activator of transcription 1 (STAT1) have been associated with chronic mucocutaneous candidiasis (CMC). This case report describes a 10-year-old boy presenting with signs of common variable immunodeficiency (CVID), failure to thrive, impaired neurological development, and a history of recurrent mucocutaneous *Candida* infections. Sequencing of the *STAT1* gene identified a heterozygous missense mutation in exon 7 encoding the STAT1 coiled-coil domain (c.514T>C, p.Phe172Leu). In addition to hypogammaglobulinemia with B-cell deficiency, and a low percentage of Th17 cells, immunological analysis of the patient revealed a marked depletion of forkhead-box P3<sup>+</sup>-expressing regulatory T cells (Tregs). *In vitro* stimulation of T cells from the patient with interferon- $\alpha$  (IFN $\alpha$ ) and/or IFN $\gamma$  resulted in a significantly increased expression of STAT1-regulated target genes such as *MIG1*, *IRF1*, *MX1*, *RIG-G*, *MCP1/CCL2*, *IFI-56K*, and *CXCL10* as compared to IFN-treated cells from a healthy control, while no IFN $\alpha$ / $\gamma$ -mediated up-regulation of the *FOXP3* gene was found. These data demonstrate that the STAT1 GOF mutation F172L, which results in impaired stability of the antiparallel STAT1 dimer conformation, is associated with inhibited Treg cell development and neurological symptoms.

## Introduction

Growing evidence suggests that several single point mutations in the gene encoding the human transcription factor STAT1 (signal transducer and activator of transcription 1) result in impaired interleukin-17 (IL-17) immunity causing chronic mucocutaneous candidiasis (CMC) (Liu et al. 2011; van de Veerdonk et al. 2011; Hori et al. 2012; Takezaki et al. 2012; Aldave et al. 2013; Soltesz et al. 2013). As

recently described, gain-of-function (GOF) mutations in the human *STAT1* gene not only cause CMC, but can also present with a variety of other clinical phenotypes due to immune dysregulation, such as intracellular dimorphic fungal (histoplasmosis and disseminated coccidioidomycosis), atypical mycobacterial infections and the wild-type forkhead-box protein 3 (FOXP3) immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)-like syndrome (Uzel et al. 2013; Sampaio et al. 2013; Kumar et al. 2014; Depner et al. 2015).

The majority of autosomal dominant, CMC-causing mutations are located at the interface between the two protomers of the antiparallel STAT1 dimer, which is formed through reciprocal binding of the coiled-coil domain of one protomer to the DNA-binding domain of its partner protomer (Zhong et al. 2005; Mertens et al., 2006; Wenta et al., 2008). Numerous studies have established that mutations affecting the stability of the antiparallel STAT1 dimer conformation result in reduced numbers of IL-17-producing CD4<sup>+</sup> T cells (Liu et al. 2011; Smeekens et al., 2011; van de Veerdonk et al. 2011; Takezaki et al. 2012; Soltesz et al. 2013). However, much less is known about the formation of regulatory T cells (Treg) in response to stimulation of cells with interferon. In this case report, we describe the clinical and laboratory findings in a STAT1-F172L mutation carrier, presenting with clinical features of CMC who exhibited low numbers of Tregs and a promoter-specific up-regulation of STAT1-driven target genes.

## **Methods**

Peripheral blood samples were taken from the patient and a healthy age- and sex-matched donor after informed consent. For phenotypic characterization, 50 µl of whole blood were incubated with antibody mixtures for 45 min and subsequently fixed and washed before analysis. The antibodies used were anti-CD3 (clone

OKT3), anti-CD4 (clones RPA-T4 or SK3), anti-CD8 (clone RPA-T8; eBioscience), anti-CD25 (clone 2A3; BD Biosciences), anti-CD127 (clone HCD127), anti-CD45RA (clone H100), anti-CCR7 (clone TG8), anti-IL-17 (clone BL168), anti-IFN $\gamma$  (clone 4S.B3; eBioscience), and anti-FOXP3 (clone 259D). All antibodies were purchased from BioLegend, if not stated otherwise. Tregs were identified as CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup> or as CD4<sup>+</sup>FOXP3<sup>+</sup>, in surface or intracellular stainings, respectively. Blood cells were first stained for cell surface molecules and then fixed and permeabilized with the appropriate buffer before being stained with anti-FOXP3. For detection of intracellular cytokines, 1x10<sup>6</sup> PBMC were stimulated for 5 h with 50 ng/ml phorbol myristate acetate, 1  $\mu$ g/ml ionomycin and IL-2 (50 U/ml; Novartis; A2542) in the presence of brefeldin A. After incubation, the samples were stained first with antibodies against surface molecules CD3 (BD; 345766), CD4 (Beckman Coulter; 737660), and CD45R0 (BD; 562299). Cells were fixed, permeabilized (BD Cytofix/Cytoperm) and stained for intracellular IFN $\gamma$  (BD; 554700), and IL-17 (eBioscience; 12-7179-42). For intracellular phospho-STAT1 staining, mononuclear cells were left either untreated or treated with IFN $\alpha$  (1000 U/ml; Miltenyi Biotec) or IFN $\gamma$  (200 ng/ml; Miltenyi Biotec) for 15 min. For detection of phospho-STAT3, cells were stimulated with IL-21 (100 ng/ml; Miltenyi Biotec) before fixation. Cells were then fixed for 10 min (Phosflow Lyse/fix buffer; BD) and stained for CD14 (IM0645U; Beckman Coulter), followed by permeabilization for 30 min (Perm III buffer; BD). Cells were additionally stained for P-STAT1 (612597; BD). To assess proliferation, freshly prepared PBMC were labeled with eFluor 670 (eBioscience) according to the manufacturer's protocols and cultured with 1  $\mu$ g/ml soluble anti-CD3 (clone OKT3; BioXcell) and 20 U/ml of IL-2. On days 3 and 4, cells were stained with anti-CD4 and anti-CD8 antibodies, and eFluor 670 dilution was measured by flow cytometry. All samples were analyzed in a FACS Canto II flow

cytometer using the FACSDiva software (BD Biosciences) or in a Navios cytometer (Beckman Coulter). Expression of STAT1-regulated target genes in T cells was assessed by means of real-time PCR. Briefly, after RNA isolation and cDNA synthesis from isolated T cells, each real-time PCR reaction was measured in duplicate in a total volume of 20  $\mu$ l, containing 1  $\mu$ l of cDNA, 70 nmol/l of each primer, and 10  $\mu$ l of Absolute Blue QPCR SYBR Green mix (Thermo Scientific). The following gene-specific primers for IFN $\alpha$ -inducible genes (*MX1*, *RIG-G*, and *IFI-56K*) were used: MX-1F: 5'-CAATCAGCCTGCTGACATTG-3', MX-1R: 5'-TGTCTCCTGCCTCTGGATG-3', RIG-GF: 5'-CAGAAGCCCAGACTTACCTG-3', RIG-GR: 5'-ATAGGCAGAGATCGCATACC-3', IFI-56KF: 5'-TAGCCAACATGTCCTCACAGAC-3', and IFI-56KR: 5'-TCTTCTACCACTGGTTTCATGC-3'. Primers used for IFN $\gamma$ -inducible genes as well as for *STAT1* and *GAPDH* were as previously described (Staab et al., 2013). The relative expression of a transcript was normalized to the expression of *GAPDH* as determined for each sample. The  $\Delta\Delta C_t$ -method was used to determine comparative relative expression levels. To compare gene expression data, unpaired Student's *t* or Mann-Whitney U tests were used, and differences were considered significant if  $p \leq 0.05$ .

## Results

### Case representation

A 10-year-old boy of non-consanguineous, healthy parents was referred to our hospital because of abdominal bloating and pain, diarrhea, weight loss and fatigue. His medical history revealed multiple *Candida* infections of the skin (face and limbs) and recurrent infections of the upper respiratory tract with frequent wheezing since early childhood, including one episode of hospital-treated pneumonia. Despite

elevated serum gliadin IgG antibodies, endoscopy showed no histological signs of celiac disease, but severe ulcerative, microbiologically confirmed *Candida albicans* esophagitis. Physical examination showed severe dystrophy (height and weight below the 3<sup>rd</sup> percentile), while endocrinological work-up showed no deficiency of growth hormone. Standardised neuropsychological tests demonstrated delayed development of speech and intellectual ability, impaired short-time memory, and a marked attention-deficit disorder.

### **Laboratory findings**

Laboratory results included anaemia (haemoglobin 9.6 g/dL), hypogammaglobulinemia and an impaired antibody production against routine vaccinations (diphtheria and tetanus). The screening test for HIV1/2 was negative. The patient had persisting B-cell lymphopenia with an increased proportion of naive (IgM<sup>+</sup>IgG<sup>D</sup>CD27<sup>-</sup>), CD21<sup>low</sup> and transitional B cells, while marginal-zone-like cells (IgD<sup>+</sup>CD27<sup>+</sup>IgM) and switched memory B and plasma cells (IgD<sup>-</sup>CD27<sup>+</sup>) were reduced. With low serum levels of IgG, IgA and IgM and a diminished response to vaccines, the patient fulfilled diagnostic criteria for common variable immunodeficiency (CVID). FACS-based functional assays demonstrated a reduction of IL-17 expression on CD4<sup>+</sup>/CD45RO<sup>+</sup> T cells (Fig. 1A). *In vitro* T-cell assays showed that *Candida* antigen stimulation resulted in a decreased IFN $\gamma$  response, while the response after phorbol myristate acetate (PMA) stimulation was high in CD4<sup>+</sup> T cells. The distribution of V beta receptor chains on CD4<sup>+</sup> and CD8<sup>+</sup> T cells appeared to be normal. In IFN $\alpha$ - and IFN $\gamma$ -stimulated monocytes from blood samples of the index patient, a significantly increased level of tyrosine-phosphorylated STAT1 was observed as compared to monocytes from a healthy control (Fig. 1B). However, the level of IL-21-induced phospho-STAT3 activation did

not differ between control and patient (Fig. 1C)

### **Genetic findings**

Using DNA sequencing, we identified a rare *de novo* (parents had wild-type genotype) heterozygous mutation (c.514T>C, p.Phe172Leu) in exon 7 of the *STAT1* gene, resulting in an amino acid exchange in the coiled-coil domain (Fig. 1D). Sanger sequencing showed no evidence of CVID-associated genetic alterations in any of the following genes tested: *BTK*, *IGHM*, *IGLL1*, *CD79A*, *CD79B*, *BLNK*, *ICOS*, *CD19*, *CD81*, *TNFRSF13B (TACI)*, and *TNFRSF13C (BAFFR)*. In addition, endocrinopathy candidiasis ectodermal dystrophy (APECED) was excluded by sequencing the *AIRE* gene (Kisand et al. 2010). Moreover, *FOXP3* was wild-type.

### **Detection of regulatory T cells and gene expression**

FACS analysis using FOXP3 staining revealed a low percentage of Tregs (Foxp3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells) as compared to control (1.9% vs 5.8%) (Fig. 2A). CD4<sup>+</sup> T cells showed normal up-regulation of the  $\alpha$ -chain of the IL-2 receptor CD25 and proliferation in response to polyclonal stimulation (Fig. 2B). Upon *in vitro* stimulation of T cells with either IFN $\alpha$  and/or IFN $\gamma$  for 6 hours, we found a markedly increased expression of *MIG1*, *MX1*, *RIG-G*, *MCP1*, *IFI-56K*, *IRF1*, and *CXCL10* genes (Fig. 3). Although the *STAT1* gene was significantly up-regulated by both IFN $\alpha$  and IFN $\gamma$  treatment, indicative of a positive feed-back loop, we found no difference in the expression level between cells from the patient carrying the GOF mutation and the healthy control.

### **Clinical course**

Medical history, clinical presentation, and results from laboratory tests led to the

clinical diagnosis of chronic mucocutaneous candidiasis (CMC) with common variable immunodeficiency (CVID), and treatment with prophylactic fluconazol, trimethoprim/sulfmethoxazole and intravenous immunoglobulins (IVIG) was initiated. During the following months, the clinical condition of the child improved and his susceptibility to upper respiratory infections declined, although he still suffered from several episodes of oral candidiasis, aphthous stomatitis and bacterial keratoconjunctivitis.

## Discussion

In the present case report, we describe the phenotype of a 10-year-old boy presenting with recurrent mucosal *Candida* infections, failure to thrive and cognitive retardation, in whom we identified a heterozygous mutation (c.514T>C, p.Phe172Leu) in *STAT1*. Immunological investigations using the patient's immune effector cells clearly demonstrated a selective T-cell defect with Th17 deficiency and a B-cell deficiency with hypogammaglobulinemia. Despite the fact that severe CMC could initially be controlled with fluconazole prophylaxis and additional supportive treatment, including antibiotic prophylaxis and intravenous immunoglobulin (IVIG) infusions, our patient still suffers from a severe clinical phenotype with dystrophy and delayed neurocognitive development. A similar mutation has been described in a 25-year-old woman born to non-consanguineous parents without a family history of fungal infections or autoimmunity, who presented with disseminated and relapsing histoplasmosis in childhood and later developed chronic and recurrent oral, cutaneous and vaginal candidiasis (Sampaio et al. 2013).

The phenylalanine residue at position 172 in the *STAT1* coiled-coil domain binds with its aromatic side chain to a pocket in the DNA-binding domain of the partner protomer located at the dimer interface which is required for the formation of



antiparallel homodimers (Zhong et al. 2005; Mertens et al., 2006; Staab et al. 2013) (Fig. 1D). Due to steric hindrance, substitution of either leucine or tryptophan for phenylalanine critically impairs the stability of the antiparallel dimer conformation and shifts the equilibrium to the parallel conformation. In the parallel conformer, the SH2 domains of the two protomers are located on the same site of the dimer and interact reciprocally *via* their phosphorylated tyrosine residues 701. Since the STAT1-inactivating tyrosine phosphatase Tc45 acts exclusively on the antiparallel dimer, a shift towards the parallel dimer conformation results in a reduced rate of tyrosine dephosphorylation and, consequently, enhanced induction of numerous IFN-driven target genes.

The clinical features of hypogammaglobulinaemia and B-cell lymphopenia found in our patient are not typical in CMC patients. In accordance with defective B-cell function, antibody production against routine vaccines such as diphtheria and tetanus was impaired. As described above, defects in *BTK*, *IGHM*, *IGLL1*, *CD79A*, *CD79B*, *BLNK*, *ICOS*, *CD19*, *CD81*, *TNFRSF13B (TACI)*, and *TNFRSF13C (BAFF-R)* that could explain the immunodeficiency in our patient have been excluded (Al-Herz et al. 2014). Recently, Romberg et al. (2013) reported on individuals of a family with a complex clinical phenotype including candidiasis, humoral immunodeficiency with hypogammaglobulinaemia and B-cell lymphopenia caused by increased B-cell apoptosis with overexpression of protein ligand 1 (PD-L1).

The pathways behind the reduced expansion of Tregs in our patient are currently unknown. Recently, Gurram and colleagues reported that treatment with the antifungal agent caerulomycin A resulted in the generation of Tregs by suppressing IFN $\gamma$ -driven STAT1 signalling *via* expression of its inhibitor SOCS1 (suppressor of cytokine signalling 1) (Gurram et al. 2013). Goodmann et al. (2011) demonstrated that the relative levels of activated STAT1 and its homolog STAT3 regulate the

effectiveness of Treg mechanisms. In the presence of highly phosphorylated STAT1, activation of STAT3 resulted in Tregs suppression, while this suppression was impaired in cells expressing low levels of activated STAT1. In a model of bone marrow transplantation, Ma et al (2011) showed that lack of STAT1 expression in donor splenocytes attenuated morbidity and mortality in graft-versus-host disease (GVHD) and resulted in the expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> donor T cells *in vivo*. The absence of STAT1 enhanced the generation of inducible Tregs by promoting their proliferation and inhibiting apoptosis of natural Tregs. However, in the case of hyperactive STAT1, this may inversely lead to low cell numbers and reduced expansion of Tregs, as was observed in our CVID patient with a dimer-specific GOF mutation.

In summary, a missense mutation in the *STAT1* gene affecting the structural stability of the antiparallel dimer formation results in a complex clinical phenotype including recurrent episodes of CMC, impaired neurocognitive development, and severe CVID. Despite unaltered proliferation of T cells in response to stimulation with anti-CD3 and IL-2, we found evidence of a reduced number of Treg cells, as judged by decreased FOXP3 staining. These clinical observations underscore the central role of STAT1 in T cell development and both cellular and humoral immunity.

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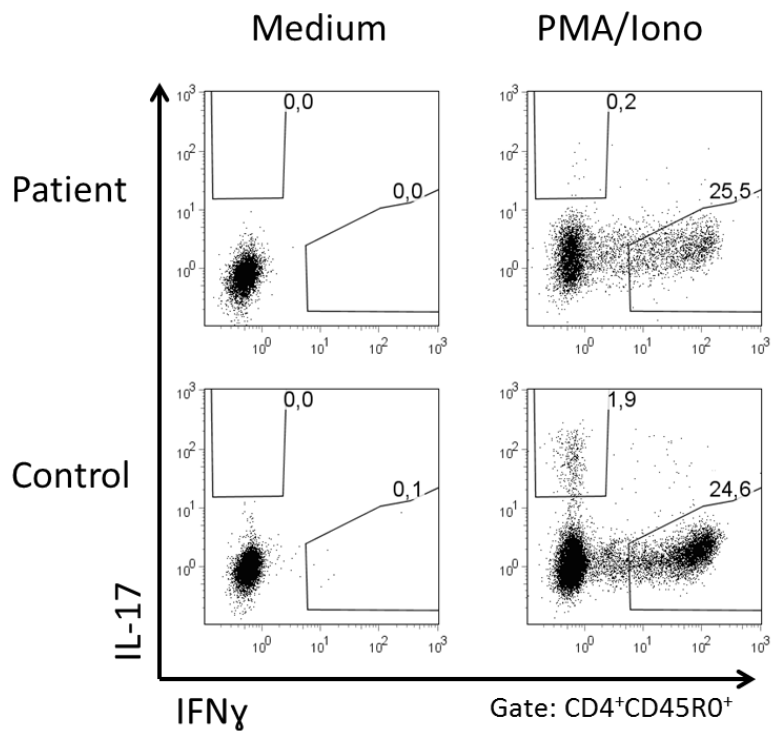


Figure 1A

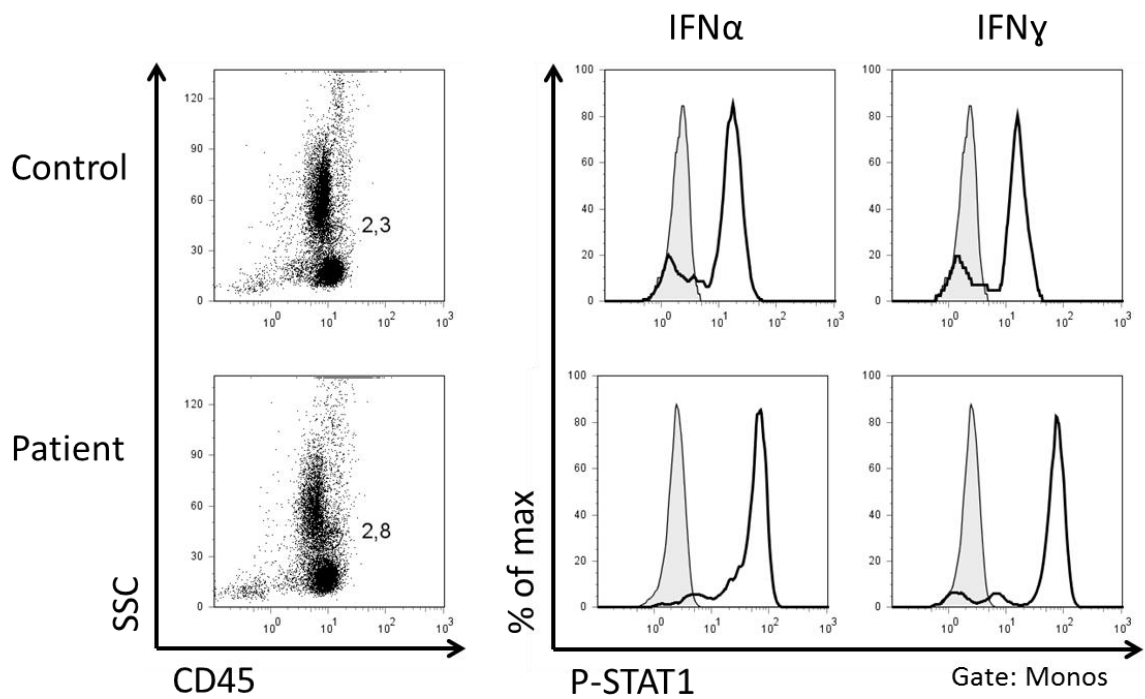


Figure 1B

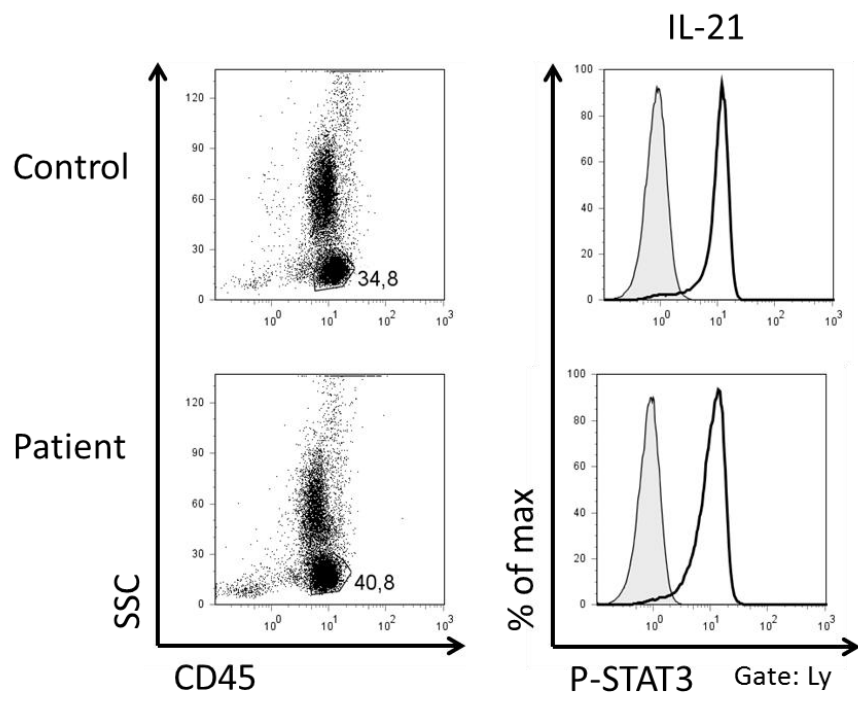


Figure 1C

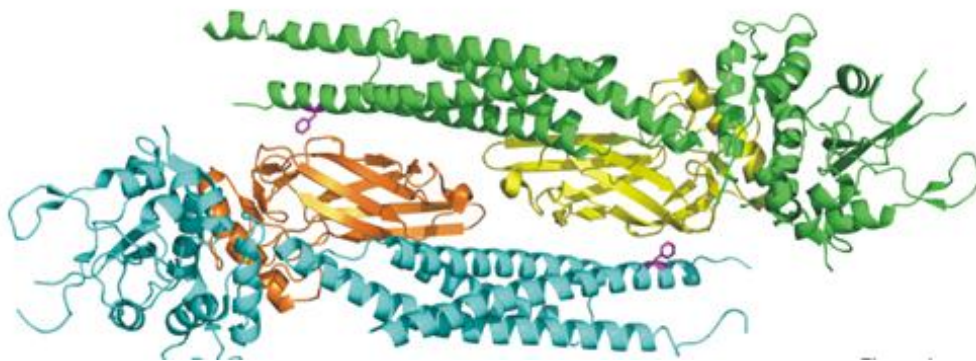


Figure 1D

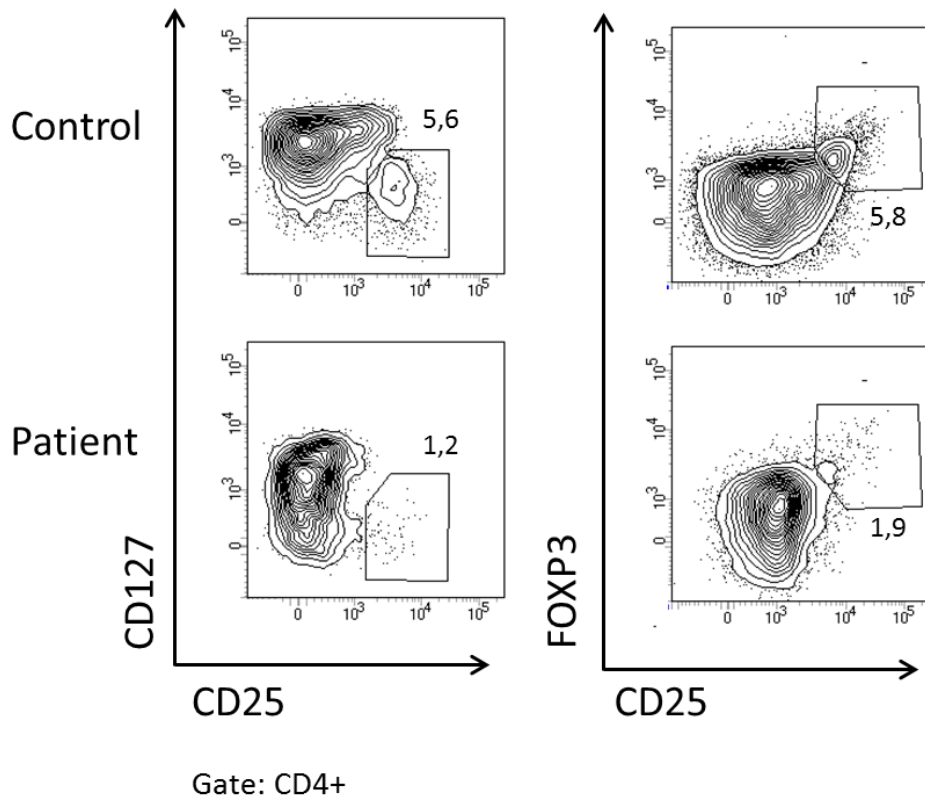


Figure 2A

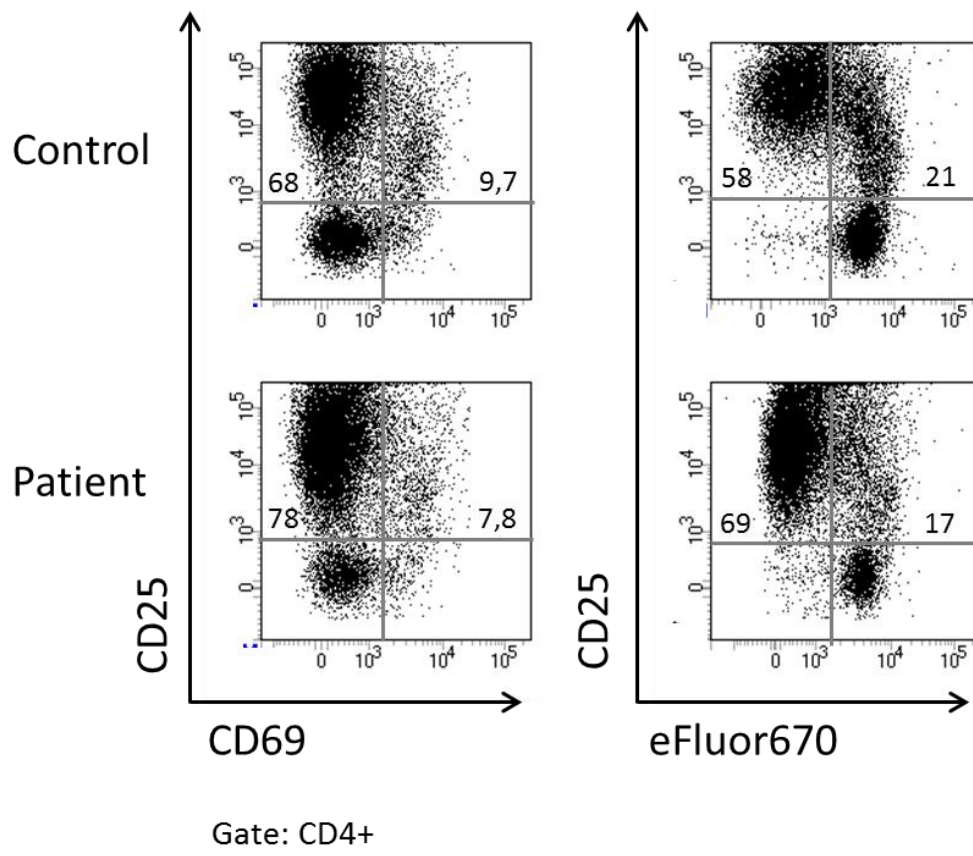


Figure 2B

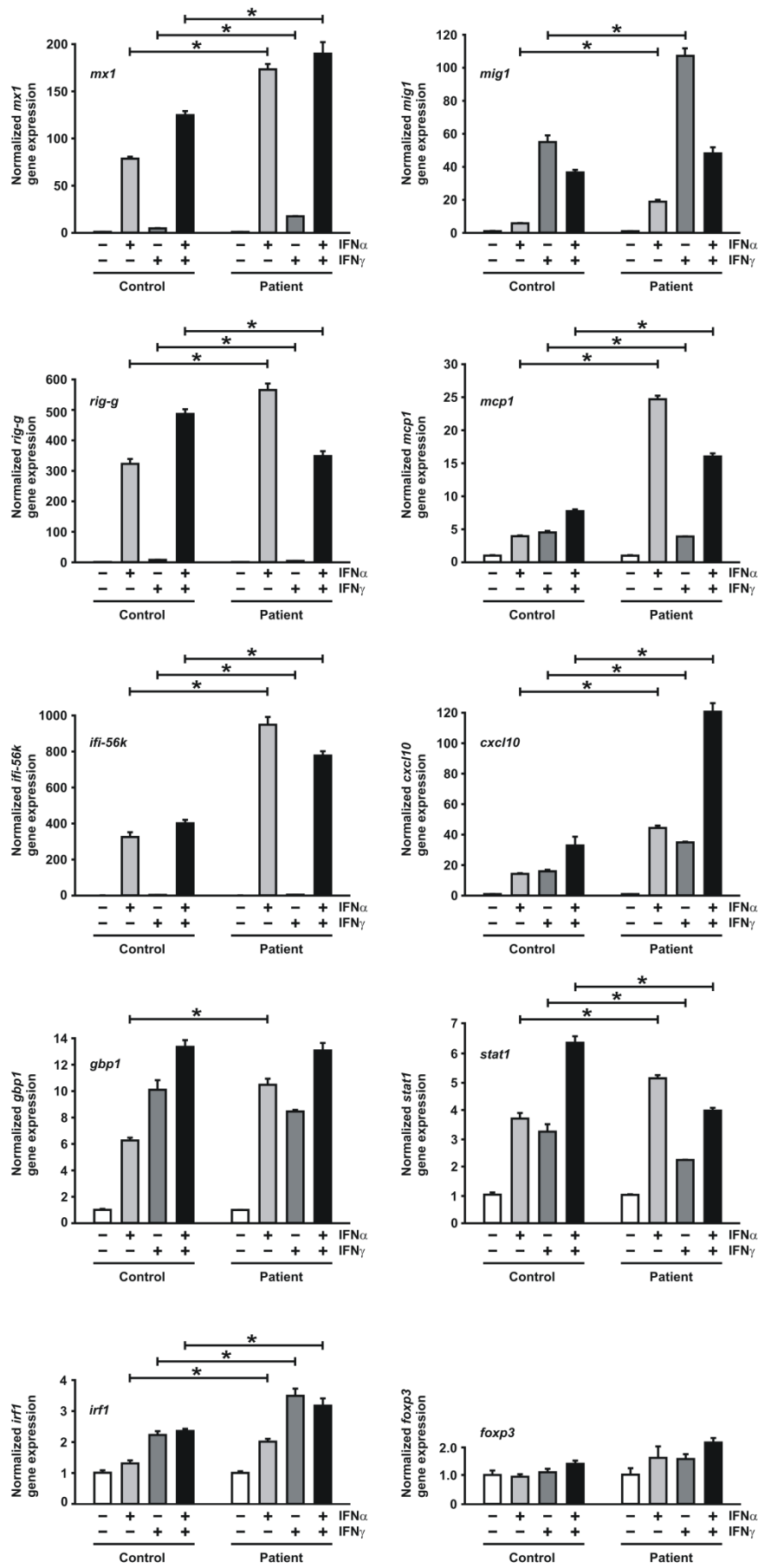


Figure 3

## Figure legends

Figure 1: Phenotypic and functional properties of lymphocytes and monocytes are indicative of STAT1 GOF. (A) Freshly isolated PBMC from the patient and a healthy control were stimulated with PMA/ionomycin in the presence of Brefeldin A for 5 h prior to intracellular staining for IL-4, IL-17 and IFN $\gamma$  (normal values: IFN $\gamma$ <sup>+</sup> of CD4<sup>+</sup>/CD45R0<sup>+</sup>: 16.4-32.6%; IL-17<sup>+</sup> of CD4<sup>+</sup>/CD45R0<sup>+</sup>: 1.1-4.7%). (B) Stimulation of monocytes with IFN $\alpha$  (500 U/ml) or IFN $\gamma$  (500 ng/ml) for 15 min followed by fixation, permeabilization and intracellular staining for tyrosine-phosphorylated STAT1 (P-STAT1) showed increased STAT1 phosphorylation in the patient's monocytes. (C) Similar pattern of IL-21-induced STAT3 phosphorylation in lymphocytes from a healthy control and the patient. (D) Crystal structure of a truncated STAT1 dimer showing the localization of the aromatic side chain of the critical phenylalanine residue 172 in the coiled-coil domain which is required for the formation of an antiparallel conformer (marked in magenta, Mao et al. 2005). The DNA-binding domain within each protomer is highlighted in a different color.

Figure 2: (A) FACS-based analysis demonstrating low percentages of regulatory T cells (1.9%) as compared to a healthy control (5.8%). (B) Normal proliferation and up-regulation of CD25 upon stimulation of CD4<sup>+</sup> T cells with anti-CD3 and IL-2.

Figure 3: Mean and standard deviation of real-time PCR data in isolated T cells from a healthy control and the patient showing gene-specific induction of STAT1-target genes before and after stimulation of T cells with IFN $\alpha$ , IFN $\gamma$  or a mixture of both cytokines. Relative gene expression was normalized to the expression of the housekeeping gene *GAPDH*.