The State of STATs in Primary Immunodeficiencies:

Molecular Diversity and Implications for Therapy

De betekenis van STATs in primaire immuundeficiëntie ziekten:

Moleculaire diversiteit en de gevolgen voor behandeling

Kornvalee Meesilpavikkai

This PhD program was supported by Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

The studies described in the thesis were performed at the Laboratory Medical Immunology, Department of Immunology, University Medical Center Rotterdam, the Netherlands

The research for this thesis was achieved in the framework of the Erasmus MC Molecular Medicine Post Graduate School

The printing of this thesis was supported by:
Erasmus MC and Department of Internal Medicine.
ISBN/EAN: 978-94-91811-23-4
Illustrations: Kornvalee Meesilpavikkai
Cover and invitation: Kornvalee Meesilpavikkai
Lay-out: Kornvalee Meesilpavikkai
Printing: Haveka BV, Hendrik-Ido-Ambacht

ISBN: 978-94-91811-23-4

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. R.C.M.E. Engels

en volgens besluit van de College voor Promoties.

De openbare verdediging zal plaatsvinden op
Woensdag 9 Oktober 2019 om 13.30
door

Kornvalee Meesilpavikkai

geboren te Bangkok, Thailand

Erasmus University Rotterdam

Promotiecommissie

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CHAPTER 2.1

Novel STAT1 gain-of-function variant

A Novel Heterozygous Mutation in the *STAT1* SH2 Domain Causes Chronic Mucocutaneous Candidiasis, Atypically Diverse Infections, Autoimmunity and Impaired Cytokine Regulation

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Front Immunol. 2017 Mar 13;8:274

Abstract

Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency (PID) characterized by persistent or recurrent skin and mucosal surface infections with *Candida* species. Different gene mutations leading to CMC have been identified. These include various heterozygous gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (*STAT1*) that are not only associated with infections but also with autoimmune manifestations. Recently, two *STAT1* GOF mutations involving the Src homology 2 (SH2) domain have been reported while so far over fifty mutations have been described mainly in the coiled-coil and the DNA binding domains. Here we present two members of a Dutch family with a novel *STAT1* mutation located in the SH2 domain. T lymphocytes of these patients revealed STAT1 hyperphosphorylation and higher expression of STAT1 target genes. The clinical picture of CMC in our patients could be explained by diminished production of interleukin (IL)-17 and IL-22, cytokines important in the protection against fungal infections.

Introduction

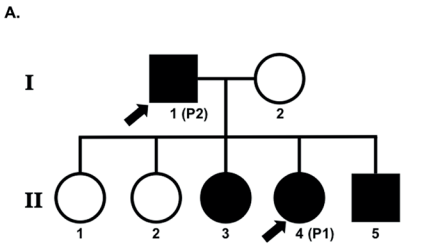
Patient 1 (II-4) (Figure 1A-B) is a 24-year-old female who presented at age 1 with a *Streptococcus haemolyticus* jaw abscess, with recurrent oral and esophageal *Candida albicans* infections from the age of 6 onwards and an episode of *Staphylococcus aureus* pneumonia and CMV pneumonia at age of 7 years. At age 8 she developed hypothyroidism and there were serologic signs of celiac disease, without clinical relevance. Moreover, additional immunological analysis revealed presence of ANA in high titer (1:5120), anti-SSA, and anti-CENP-B autoantibodies. At the age of 20 she developed autoimmune hemolytic anemia. Over the past years, the clinical picture has been dominated by recurrent oral and esophageal *Candida albicans* infections for which she was repetitively treated with antifungal therapies. To prevent recurrence of *Candida albicans* infections she is currently treated with prophylactic antifungal therapy (fluconazole 200 mg once daily). Moreover, she has experienced oral and vaginal ulcers which were found negative for bacterial, fungal and viral microbes as determined by culture and/or PCR of oral and vaginal swabs and tissue biopsies from vaginal ulcers. Because of these ulcers, which were assumed to be autoimmune manifestations related to CMC, various immunosuppressive therapies have been initiated over time, including steroids, azathioprine, hydroxychloroquine, mycophenolate mofetil, all with little benefit. She has recently started treatment with adalimumab (anti-tumor necrosis factor (TNF)-α) 40 mg

every other week, which resulted in complete resolution of oral and vaginal ulcers after 3 subcutaneous injections. After three months of treatment, there is still no recurrence of oral and/or vaginal ulcers, whereas no increased incidence of infectious complications was reported while using anti-TNF-α treatment.

Peripheral blood total CD3+ T lymphocyte numbers (1.63×10^9/L [reference value: 0.7-2.1$\times10^9$/L]), CD4+ T lymphocyte numbers (0.6×10^9/L [reference value: 0.3-1.4$\times10^9$/L]), CD8+ T lymphocyte numbers (0.84×10^9/L [reference value: 0.2-0.9$\times10^9$/L]), total CD19+ B lymphocyte numbers (0.16×10^9/L [reference value: 0.1-0.4$\times10^9$/L]) and CD16+ CD56+ NK cell numbers (0.1×10^9/L [reference value: 0.1-0.4$\times10^9$/L]) were within the normal reference ranges. Immunoglobulin levels were also found to be within normal limits (IgG 10.5 g/l [reference value: 7.0-16.0 g/l], IgA 1.24 g/l [reference value: 0.76-3.91 g/l] and IgM 1.12 g/l [reference value: 0.45-2.30 g/l]). The patient was negative for autoantibodies against IL-17A, IL-17F and IL-22. After immunization with a polysaccharide vaccine against *Streptococcus pneumonia* (Pneumovax), IgG antibody concentration to all 16 serotypes (1, 3, 4, 5, 6B, 7F, 8, 9V, 14, 15B, 18C, 19A, 19F, 20, 23F, and 33F) measured increased when compared to pre-vaccination concentrations and for 12 serotypes the post-immunization concentrations reached values above 1 μg/ml, which is indicative of a normal response to polysaccharide immunization[1].

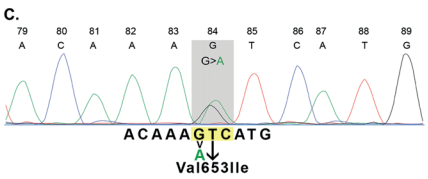
Patient 2 (I-1) (Figure 1A-B) is the 50-year-old father of patient 1, with a medical history including aortic valve replacement at age 29 and surgical and antibiotic treatment for a culture-negative, frontal-lobe brain abscess at age 33. He presented for the first time at our outpatient clinic at the age of 49 because of very severe *Candida albicans* infection in the oral cavity and esophagus. At that time he had already suffered from recurrent oral and esophageal fungal infections for years, for which he was treated with antifungal therapies by his general physician. Microbiological analysis revealed a fluconazole-resistant *Candida albicans* and treatment with posaconazole 400 mg once daily was initiated with prompt clinical improvement. Subsequently, prophylactic posaconazole 200 mg once daily has been prescribed. At time of first analysis, also autoimmune hypothyroidism with positivity for anti-thyroglobulin antibodies, ANA and lupus anticoagulant were detected. Peripheral blood total CD3+ T lymphocyte numbers (1.06×10^9/L [reference value: 0.7-2.1$\times10^9$/L]), CD4+ T lymphocyte numbers (0.5×10^9/L [reference value: 0.3-1.4$\times10^9$/L]), CD8+ T lymphocyte numbers (0.52×10^9/L [reference value: 0.2-0.9$\times10^9$/L]), total CD19+ B lymphocyte numbers (0.35×10^9/L [reference value: 0.1-0.4$\times10^9$/L]) and

CD16+ CD56+ NK cell numbers (0.16×10^9/L [reference value: 0.1-0.4$\times10^9$/L]) were within the normal reference range. Immunoglobulin levels were also found within normal limits (IgG 12.9 g/l [reference value: 7.0-16.0 g/l], IgA 1.65 g/l [reference value: 0.76-3.91 g/l] and IgM 0.8 g/l [reference value: 0.45-2.30 g/l]). The patient was negative for autoantibodies against IL-17A, IL-17F and IL-22.



B.

Patient (age)	Clinical features	Laboratory findings
I-1 (50)	Recurrent mucosal candidiasis, culture-negative frontal-lobe brain abscess, aortic valve replacement	ANA+, anti-CENP-B+, anti-thyroglobulin+ lupus anticoagulant+
II-3 (25)	Recurrent mucosal candidiasis, ulcerative stomatitis and esophagitis, photosensitivity, skin lesions	ANA+, anti-dsDNA+, anti-gliadin antibodies+, lupus anticoagulant+
II-4 (24)	Recurrent mucosal candidiasis, ulcerative stomatitis and esophagitis, vaginal ulcers, hypothyroidism, hemolytic anemia	ANA+, anti-SSA+, Anti-CENP-B+, anti-gliadin IgA/IgG+
II-5 (13)	Recurrent mucosal candidiasis, ulcerative stomatitis and esophagitis, *Staphylococcus aureus* pneumonia	Anti-gliadin IgG+



D.

Organisms		Sequence features
H. sapiens	637	KELSAVTFPDIIRNYK**V**MAAENIPENPLKYLYPNIDKD
P. troglodytes	639	KELSAVTFPDIIRNYK**V**MAAENIPENPLKYLYPNIDKD
M. mulatta	637	KELSAVTFPDIIRNYK**V**MAAENIPENPLKFLYPNIDKD
C. lupus	637	KELSAVTFPDIIRNYK**V**MAAENIPENPLKFLYPNIDKD
B. taurus	637	KELSAVTFPDIIRNYK**V**MAAENIPENPLKFLYPNIDKD
M. musculus	643	KELSAVTFPDIIRNYK**V**MAAENIPENPLKYLYPNIDKD
R. norvegicus	637	KELSAVTFPDIIRNYK**V**MAAENIPENPLKYLYPNIDKD
G. gallus	639	KELSAVTFPDIIRNYK**V**MAAENIPENPLRFLYPDIPKD
D. rerio	634	KELSAVSLPDIIRNYR**V**MAADNVPENPLRFLYPQIPKD

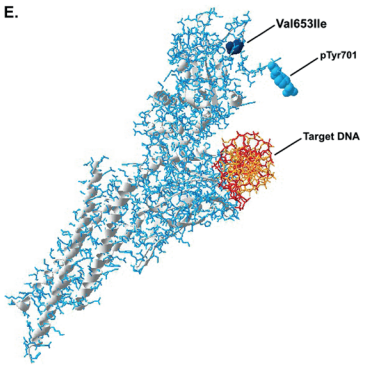


Figure 1. (A) Family pedigree of patients. Symbols in black indicate individuals with the same genetic defect and arrow signs indicate the patients enrolled in this study. **(B)** Table showing clinical data of all four affected individuals. **(C)** Sanger DNA sequencing chromatogram of mutated STAT1 gene. **(D)** Evolutionary conservation of p.Val653 among species. **(E)** Three-dimensional structure of phosphorylated STAT1 protein with the mutation (Val653Ile), the phosphorylation site (pTyr701) and the target DNA indicated.

Sanger sequencing from all affected individuals in the family revealed a novel heterozygous *STAT1* mutation in exon 22 at c.1957G>A, while genetic testing for mutations in 276 other known genes involved in PID was negative (Table 1). The nucleotide base change we identified has not been reported as single nucleotide polymorphism (the Human Gene Mutation Database, the National Center of Biotechnology Information, the ExAC database, the 1000G database and the Ensembl database). The identified mutation results in replacement of a highly conserved valine at position 653 (vertebrate Phylop100 score 3.798 and SiPhy score 19.656) (Figure 1C-D) into isoleucine (p.(Val653Ile)) within the SH2 domain of STAT1. The mutation is exposed on the outer surface of the molecule and within the vicinity of the phosphorylation site (Figure 1E). The Val653Ile mutation is predicted to hardly affect the overall structure of STAT1 and was predicted as 'tolerated' by the Sort Intolerant From Tolerant (SIFT) algorithm (score 0.58). However, Val653Ile was predicted as 'possibly damaging' by the Polymorphism Phenotyping v2 (PolyPhen-2, score 0.919), the mutation was speculated as 'disease causing' from the Mutation Taster and was scaled in Combined Annotation Dependent Depletion (CADD) algorithm with a score of 14.99, which suggests potential deleteriousness.

To evaluate the immunological phenotype associated with this mutation, STAT1 phosphorylation was studied by flow cytometry of fresh whole blood samples from patient 1 and an age-gender-race-matched healthy control. After stimulation with interferon (IFN)-α (10^4 IU/ml; PeproTech, London, UK), IFN-β (10^3 IU/ml; tebu-bio, Le-Perray-en-Yvelines, France), IFN-g (10^5 IU/ml; R&D systems, Abingdon, UK) for 30 minutes or IL-6 (100 ng/ml; R&D systems) for 15 minutes, cells were fixed and permeabilized with permeabilizing reagent (Phospho-Epitopes Exposure kit; Beckman Coulter). All samples were stained with CD3 (APC-conjugated anti-human CD3; BD Biosciences, California, USA) and Fluor® 488-conjugated phospho-STAT1 Tyr701 antibodies (Cell Signaling Technology, Massachusetts, USA). The levels of STAT1 phosphorylation in the CD3+ T lymphocytes from the patient were higher than the levels observed in the healthy control after stimulation with IFNs (Figure 2A) or IL-6 (data not shown). To study the kinetics of STAT1 phosphorylation in more detail, time-course stimulation experiments were performed with fresh whole blood samples from patient 1 and patient 2 along with age-gender-race-matched healthy controls. The patients clearly displayed higher levels of STAT1 phosphorylation, yet the phosphorylation levels of both the patients and controls normalized over time (Figure 2B).

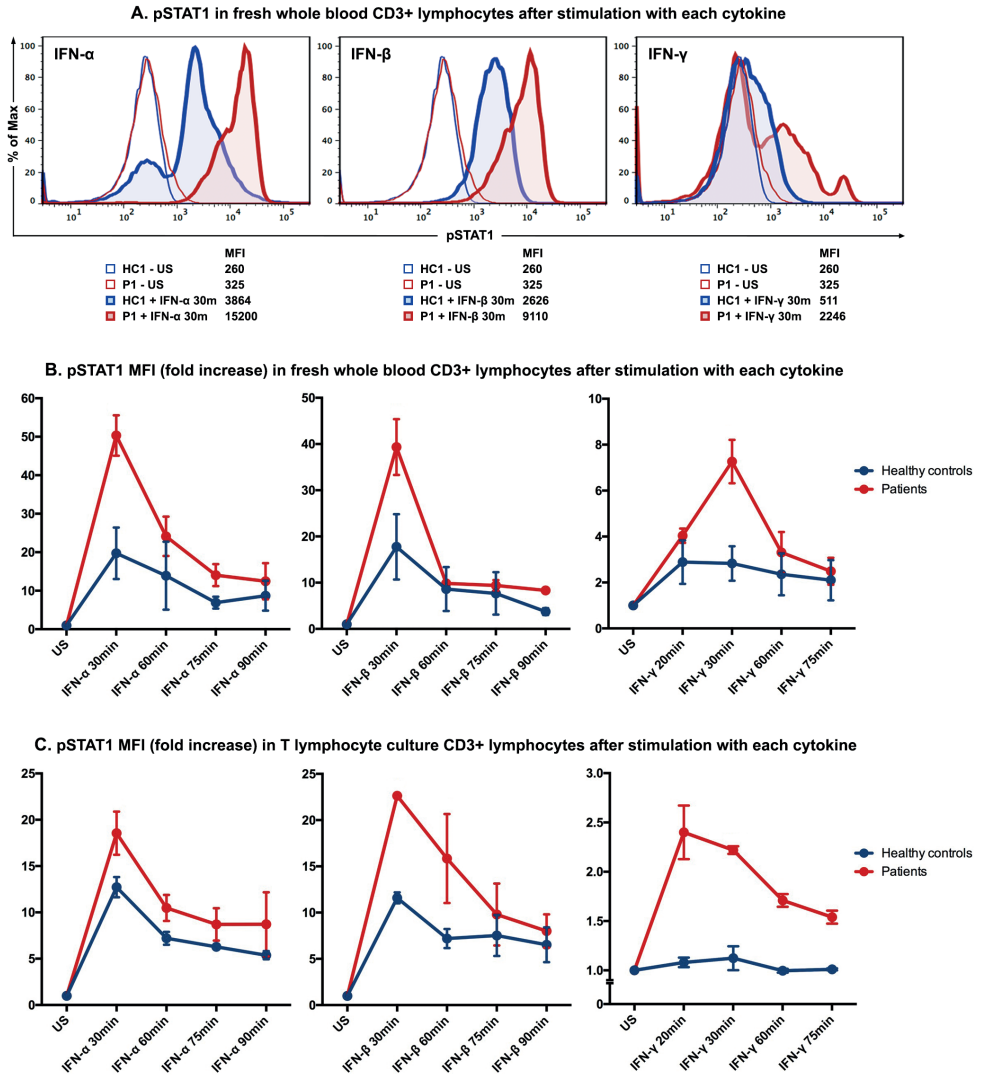


Figure 2. STAT1 phosphorylation evaluated by intracellular staining flow cytometry after stimulation with IFN-α, IFN-β or IFN-γ. **(A)** Histograms showing MFI of phosphorylated STAT1 (pSTAT1) in CD3+ T lymphocytes (in fresh whole blood) of patient P1 and healthy control (HC1) after stimulation with IFN-α, IFN-β or IFN-γ for 30 minutes. **(B)** Kinetics of STAT1 phosphorylation in CD3+ T lymphocytes (in fresh whole blood) of the two patients and two healthy controls after stimulation with IFN-α, IFN-β or IFN-γ. **(C)** Kinetics of STAT1 phosphorylation in T lymphocyte cultures from both patients and healthy controls. HC, healthy control; P, patient; US, unstimulated; MFI, mean fluorescence intensity.

Table 1 List of PID causing genes evaluated in the affected individuals

ACP5	*CD59*	*GATA2*	*MS4A1*	*RORC*	*TRAF3*
ACTB	*CD79A*	*GFI1*	*MSH6*	*RPSA*	*TRAF3IP2*
ADA	*CD79B*	*HAX1*	*MTHFD1*	*RTEL1*	*TREX1*
ADAR	*CD81*	*ICOS*	*MVK*	*SAMHD1*	*TRNT1A*
AICDA	*CD8A*	*IFIH1*	*MYD88*	*SBDS*	*TTC7A*
AIRE	*CEBPE*	*IFNGR1*	*NBN*	*SEMA3E*	*TYK2*
AK2	*CECR1*	*IFNGR2*	*NCF1*	*SERPING1*	*UNC119*
AP3B1	*CFB*	*IGLL1*	*NCF2*	*SH2D1A*	*UNC13D*
APOL1	*CFD*	*IKBKB*	*NCF4*	*SH3BP2*	*UNC93B1*
ATM	*CFH*	*IKBKG*	*NFAT5*	*SLC29A3*	*UNG*
B2M	*CFHR1*	*IKZF1*	*NFKB2*	*SLC35C1*	*USB1*
BCL10	*CFHR2*	*IL10*	*NFKBIA*	*SLC37A4*	*VPS13B*
BLM	*CFHR3*	*IL10RA*	*NHEJ1*	*SLC46A1*	*VPS45*
BLNK	*CFHR4*	*IL10RB*	*NHP2*	*SMARCAL1*	*WAS*
BTK	*CFHR5*	*IL12B*	*NLRP12*	*SP110*	*WIPF1*
C1QA	*CFI*	*IL12RB1*	*NLRP3*	*SPINK5*	*XIAP*
C1QB	*CFP*	*IL17F*	*NOD2*	*STAT1*	*XLF*
C1QC	*CHD7*	*IL17RA*	*NOP10*	*STAT2*	*XRCC4*
C1R	*CIITA*	*IL17RC*	*NRAS*	*STAT3*	*XRCC5*
C1S	*CLPB*	*IL1RN*	*ORAI1*	*STAT5B*	*XRCC6*
C2	*COH1*	*IL21R*	*OX40*	*STIM1*	*ZAP70*
C3	*COLEC11*	*IL2RA*	*PARN*	*STK4*	*ZBTB24*
C4A	*COPA*	*IL2RG*	*PGM3*	*STX11*	
C4B	*CORO1A*	*IL36RN*	*PIK3CD*	*STXBP2*	
C5	*CR2*	*IL7R*	*PIK3R1*	*TAP1*	
C6	*CR3*	*INO80*	*PLCG2*	*TAP2*	
C7	*CSF2RA*	*IRAK4*	*PLDN*	*TAPBP*	
C8A	*CSF3R*	*IRF7*	*PMS2*	*TAZ*	
C8B	*CTPS1*	*IRF8*	*PNP*	*TBK1*	
C8G	*CTSC*	*ISG15*	*POLE*	*TBX1*	
C9	*CXCR4*	*ITCH*	*PRF1*	*TCF3*	
C9orf142	*CYBA*	*ITGB2*	*PRKCD*	*TCN2*	
CARD11	*CYBB*	*ITK*	*PRKDC*	*TERC*	
CARD14	*DCLRE1B*	*JAGN1*	*PSMB8*	*TERT*	
CARD9	*DCLRE1C*	*JAK3*	*PSTPIP1*	*THBD*	
CASP10	*DKC1*	*KINDLIN-3*	*PTPRC*	*TICAM1*	
CASP8	*DNMT3B*	*KRAS*	*RAB27A*	*TINF2*	
CCBE1	*DOCK8*	*LAMTOR2*	*RAC2*	*TLR3*	
CD16	*DPKC*	*LCK*	*RAG1*	*TMC6*	
CD19	*ELANE*	*LIG4*	*RAG2*	*TMC8*	
CD20	*EPG5*	*LPIN2*	*RBCK1*	*TMEM173*	
CD21	*FADD*	*LRBA*	*RFX5*	*TNFRSF13B*	
CD247	*FAS*	*LYST*	*RFXANK*	*TNFRSF13C*	
CD27	*FASLG*	*MAGT1*	*RFXAP*	*TNFRSF1A*	
CD3D	*FCN3*	*MALT1*	*RHOH*	*TNFRSF4*	
CD3E	*FERMT3*	*MAP3K14*	*RMRP*	*TNFRSF6*	
CD3G	*FOXN1*	*MASP1*	*RNASEH2A*	*TNFSF12*	
CD3Z	*FOXP3*	*MASP2*	*RNASEH2B*	*TNFSF6*	
CD40	*FPR1*	*MCM4*	*RNASEH2C*	*TPP1*	
CD40LG	*G6PC3*	*MEFV*	*RNF168*	*TPP2*	
CD46	*G6PT1*	*MRE11A*	*RNF31*	*TRAC*	

Because we found a higher basal level of phosphorylated STAT1 in patient 1 (Figure 3) we established T lymphocyte cultures to reduce the confounding effects of exposure of both patients to different pathogens and therapies. Long-term T lymphocyte cultures were expanded from peripheral blood mononuclear cells (PBMC) of both patients and two age-gender-race matched healthy controls in RPMI-1640 medium (Lonza, Basel, Switzerland) containing 10% heat inactivated human serum and antibiotics (2% penicillin and streptomycin; Cambrex BioWhittaker, Verviers, Belgium) in the presence of Phytohemagglutinin PHA-P (1 μg/ml; Sigma-Aldrich, Missouri, USA), IL-2 (25 IU/ml; Novartis, Basel, Switzerland), IL-15 (12.5 ng/ml; BioLegend, California, USA) and γ-irradiated (40 Gy) allogeneic PBMC and EBV-negative B lymphocytes. After 2 weeks of culturing, T lymphocyte cultures (purity > 90%) from both patients and healthy controls were analyzed for STAT1 phosphorylation in a manner similar to the whole blood samples. IFN stimulation resulted in higher generation of phosphorylated STAT1 (pSTAT1) in the T lymphocyte cultures from patients compared to healthy controls (Figure 2C). Stimulation of T lymphocyte cultures with IL-6 (100 ng/ml) yielded comparable results on pSTAT1 as IFN-γ (data not shown). T lymphocytes from the patients and healthy controls displayed similar levels of total STAT1 protein (determined by flow cytometry using a PE-conjugated STAT1 antibody (BD Biosciences; data not shown)). STAT3 phosphorylation was also evaluated after stimulation with IL-6 (100 ng/ml) or IL-21 (200 ng/ml; Life Technologies, Massachusetts, USA) but did not differ between patients and healthy controls (data not shown).

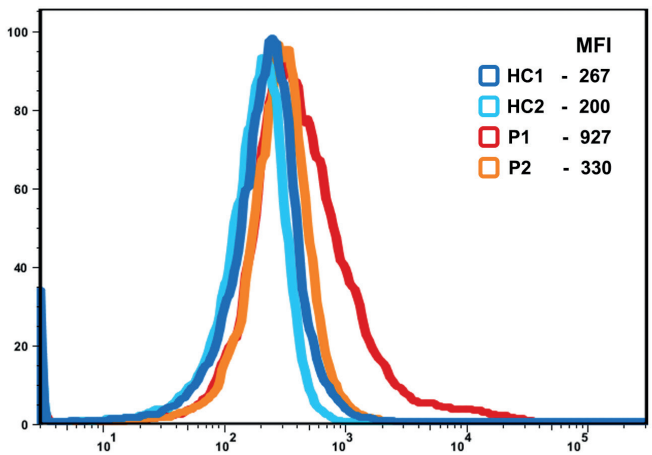


Figure 3. Histogram showing MFI of phosphorylated STAT1 in CD3+ T lymphocytes evaluated by intracellular staining flow cytometry (in fresh whole blood) at basal level. HC, healthy control; P, patient; MFI, mean fluorescence intensity.

To assess the effect of the *STAT1* mutation on cytokine production, PBMC of both patients and five race-matched healthy controls were freshly prepared and stimulated with heat-killed *Candida albicans* (HKCA: 10^6 cells). Supernatants were collected and levels of cytokines were measured by ELISA (R&D systems). Cells were also stimulated with phorbol 12-myristate 13-acetate (PMA) (81 nM) and ionomycin (I) (1.3 μM; eBioscience, California, USA) as positive control. PBMC from both patients produced IL-6 and IL-1β levels comparable to that of healthy controls, while PBMC from patient 1 did not produce IFN-g when stimulated with HKCA (Figure 4A). In contrast to PBMC from healthy controls, PBMC from the patients hardly increased IL-17A, IL-17F and IL-22 production upon stimulation with PMA-ionomycin or HKCA (Figure 4A). STAT1 downstream target genes were also measured in T lymphocyte cultures. Due to limited T lymphocyte numbers in these cultures, only one time-point of stimulation (24 hours) with three different stimuli (IFN-g (10^5 IU/ml), IL-6 (100 ng/ml), IL-27 (200 ng/ml; R&D systems)) was examined. *CXCL9*, *CXCL10* and *CD274* (PD-L1) mRNA expression levels were determined by real-time quantitative Taqman PCR in each sample on the basis of 6 replicates. Strikingly high mRNA levels were observed after IL-27 activation ($P < 0.001$) (Figure 4B).

Background

Chronic and/or recurrent fungal infections may be predominant manifestations in primary immunodeficiencies, especially in inherited T lymphocyte defects. Various syndromes have been identified that present with recurrent fungal infections, for example, autosomal dominant (AD) hyper-immunoglobulin E (IgE) syndrome (AD-HIES), autosomal recessive (AR) autoimmune polyendocrine syndrome type I (APS-1), and Mendelian susceptibility to mycobacterial diseases (MSMD). AD-CMC is a rare and severe immunodeficiency that presents with severe mucocutaneous fungal infections, autoimmune phenomena, cerebral aneurysms and increased risk of oropharyngeal and esophageal cancer[2-4]. In the last decade, various heterozygous GOF mutations in *STAT1* were found to be responsible for AD-CMC. So far, GOF mutations were described in the coiled-coil domain and the DNA binding domain of STAT1 and in the past year, the GOF mutations c.1885C>T (p.H629Y) and c.1973G>A (p.N658S) involving the SH2 domain were described[5-7]. Chronic and/or recurrent mucocutaneous fungal infections with predominantly *Candida albicans* are the major infectious complications in patients with *STAT1* GOF mutations and generally arise in infancy or childhood. However, more than half of

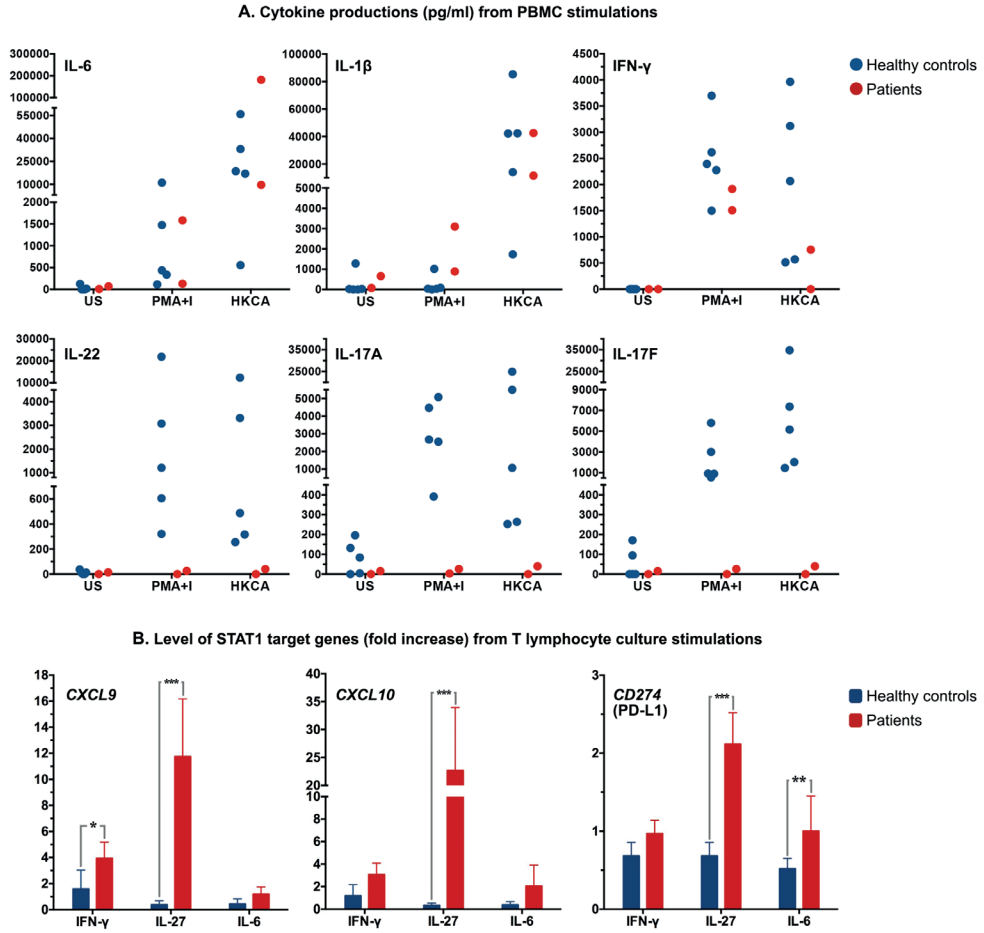


Figure 4. (A) Dot plots depicting cytokine production in supernatant from peripheral blood mononuclear cells (PBMC) from both patients and healthy controls (n = 5) after stimulation of 106 PBMC with PMA-ionomycin (PMA+I) or heat-killed *C. albicans* (HKCA). Every symbol indicates an individual. US = unstimulated. **(B)** T lymphocyte cultures were stimulated for 24 hours with IFN-γ, IL-27 or IL-6 and mRNA expression levels of *CXCL9*, *CXCL10* and *CD274* (PD-L1) were determined by real-time quantitative Taqman PCR. Data were normalized to the housekeeping gene, ABL. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with healthy controls.

the patients encounter bacterial infections, with lower respiratory tract infections most frequently observed. Cutaneous viral infections are also described in about one third of patients[6,8]. In addition, many patients with GOF mutations in *STAT1* develop autoimmune manifestations. Autoimmune thyroid disease is reported in twenty-two percent of patients. Cerebral aneurysms and cancers are among the most severe complications that are found more frequently and at younger age in these patients when compared to the general population[6,8].

Discussion

The mutation identified in our patients is located in the *STAT1* SH2 domain resulting in recurrent mucocutaneous *Candida albicans* infections, which is one of the clinical hallmarks of CMC. The other two family members that carry the same STAT1 mutation but were not included in this study also suffered from recurrent mucocutaneous candidiasis (Figure 1B). In addition to fungal infections, patient 1 showed an atypical susceptibility to a wide range of pathogens as she experienced *Streptococcus haemolyticus* jaw abscess, *Staphylococcus aureus* pneumonia and CMV pneumonia. Lower respiratory tract bacterial infections can be found in about half of patients with *STAT1* GOF mutations. However, based on previous reports, CMV pneumonia was described in only one percent of patients. Patient 2 also encountered an intracranial abscess for which surgical and antibiotic treatments were required. Although the pathogenic microorganism could not be isolated, invasive deep-seated abscesses are uncommon for patients with *STAT1* GOF mutations[6]. Apart from CMC, atypical features of infections including invasive bacterial infections were also described in other patients carrying a *STAT1* GOF mutation in the SH2 domain[5-7]. Both patient 1 and patient 2 enrolled in this study developed a variety of autoimmune phenomena that were displayed in both clinical and laboratory analysis.

In response to various kinds of infections, STAT1 receives signals from the cell surface receptors and is subsequently phosphorylated at Tyr701. The SH2 domain carries the pTyr701-binding site and accordingly plays an important role in forming a firm cross-linkage dimerization between each STAT1 monomer. The dimers then accumulate in the nucleus, inducing transcription of genes[9]. The mutation here described within the SH2 domain of STAT1 results in enhancement of STAT1 phosphorylation both upon stimulation with IFN-α/β and IFN-γ, despite the suggestion that the SH2 domain may not necessarily be required for STAT1 activation by IFN-α/β[10]. Moreover, this mutation may possibly affect molecule dimerization due to its specific location within the STAT1 molecule.

We performed STAT phosphorylation analysis of both patients by flow cytometry which revealed enhancement of STAT1 phosphorylation while total STAT1 protein was equally measured in T lymphocytes of both patients and healthy controls. After stimulation with cytokines, STAT1 phosphorylation in the T lymphocytes of both patients reached higher levels compared to the healthy controls. In contrast to previous reports studying mutations in other STAT1 domains[11-13], we found no clear prolonged STAT1 phosphorylation in the T lymphocytes from the patients. The difference in the mutated domain of *STAT1* could possibly be the cause of different phosphorylation characteristics. Apart from elevated pSTAT1 induction, we also noticed a higher basal pSTAT1 level in peripheral blood T lymphocytes from patient 1. Stimulation of fresh whole blood from patient 2 yielded comparable results, although this patient showed no elevated basal level of pSTAT1 when compared to healthy controls. However, after re-evaluation in established T lymphocyte cultures, the elevated basal pSTAT1 level previously noted in the whole blood analysis of patient 1 disappeared. Due to the fact that patient 2 received prophylactic antifungal therapy while patient 1 did not at time of analysis, recurrent exposure of patient 1 to *Candida* antigens could have caused the elevated basal STAT1 phosphorylation we observed in her whole blood analysis. Because dysregulation of STAT3 has also been associated with CMC, we evaluated STAT3 phosphorylation as well, but this was comparable between the patients and healthy controls.

Th17-derived cytokines are crucial in fungal defense mechanisms[14,15] and therefore we examined the cytokine production capacity of PBMC of both patients as well as healthy controls upon activation with HKCA. A remarkable impairment in IL-17A, IL-17F and IL-22 production was found in the patients. In order to further examine the consequence of this novel *STAT1* SH2 domain mutation, STAT1 downstream target genes were assessed. T lymphocyte cultures from both patients and healthy controls were stimulated with IFN-γ, IL-6, and IL-27. Significantly higher mRNA levels of STAT1 downstream target genes were found in both patients, especially upon IL-27 activation, including *CD274* (PD-L1). Overexpression of PD-L1 was previously observed in naïve T lymphocytes of patients with *STAT1* GOF mutation and cytokine-induced PD-L1 expression in T lymphocytes was found to hamper Th17 induction[16,17]. Our data suggest that disturbed Th17 differentiation and associated cytokine production most likely underlies the clinical picture of CMC in the patients here described.

Since curative treatment is still unavailable, most of the patients with *STAT1*

GOF mutations receive prolonged systemic antimicrobial medications to control clinical symptoms of recurrent fungal infections and other infections. Similar to patient 2, about forty percent of the patients who require long-term antifungal treatment, eventually develop therapy resistance[6]. Immunotherapies or immunosuppressive therapies are considered in some patients, although the effectiveness still needs to be evaluated in more detail[18]. Surprisingly, novel therapies, like JAK1/2 inhibitors showed not only the potency to suppress the enhanced STAT1 phosphorylation in CD4+ T lymphocytes but may also improve clinical outcome in both immunodeficiency and autoimmunity features[13,19]. These drugs could be potential future candidates for the treatment of patients with CMC with *STAT1* GOF mutations.

Concluding Remarks

The novel Val653Ile mutation, located in the SH2 domain of STAT1 found in this family does not clearly result in an impaired STAT1 dephosphorylation rate, as was found in patients with GOF mutations in the other domains. However, significantly enhanced STAT1 phosphorylation in these patients results in higher expression of the STAT1 target genes *CXCL9*, *CXCL10* and *CD274* (PD-L1). Moreover, this mutation is associated with the impairment of immune cells to produce IL-17A, IL-17F and IL-22. The clinical symptoms of CMC could therefore be explained by diminished Th17 responses that are crucial for confronting fungal antigens.

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CHAPTER 2.2

Baricitinib treatment in the patient with STAT1 gain-of-function variant

Baricitinib Treatment in a patient with gain-of-function mutation in signal transducer and activator of transcription 1 (*STAT1*)

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J Allergy Clin Immunol. 2018 Jul;142(1):328-330.e2

To the Editor:

Heterozygous gain-of-function (GOF) mutations in signal transducer and activator of transcription 1 (*STAT1*) have been increasingly reported worldwide[1]. Chronic mucocutaneous candidiasis (CMC) is the hallmark of *STAT1* GOF mutations, but bacterial infections, mainly caused by *Staphylococcus aureus,* viral infections, predominantly *Herpesviridae*, and autoimmune manifestations are also commonly present[1]. Enhanced STAT1 phosphorylation in patients with *STAT1* GOF mutations is associated with overexpression of programmed death ligand 1 (PD-L1) and abolishes T-helper 17 (Th17) responses, which is considered to represent the immunological cause of CMC[2-4].

Treatment of CMC in *STAT1* GOF patients includes long-term systemic antifungal therapy[1]. Considering the underlying immunologic defect, immunomodulatory treatment options are also explored, although sparsely and their effectiveness is still indecisive[5]. The Janus kinase (JAK) 1/2 inhibitor, ruxolitinib was reported beneficial in one patient, but increased IL-17 production was not demonstrated in this case[6]. Baricitinib is a novel, orally available JAK 1/2 inhibitor that hampers interferon (IFN) induced JAK-STAT1 signalling in immune-mediated diseases and was recently approved for treatment of rheumatoid arthritis[7]. Based on its mechanisms of action it was hypothesized that baricitinib could be of benefit in the treatment of *STAT1* GOF patients. In this report, we describe a patient treated with baricitinib and show its potential clinical implications for treatment of patients with *STAT1* GOF mutation.

The patient is a 24-year-old Dutch female diagnosed with CMC based on heterozygous *STAT1* GOF mutation at c.1957G>A (p.(V653I)) of the Src homology (SH) 2 domain. She also experienced other infectious complications and autoimmune phenomena, as described in detail previously[4]. Over the years, main clinical problems included recurrent oral and esophageal *Candida albicans* infections. Candidiasis repetitively erupted within two weeks after termination of a course of antifungal therapy. Therefore, prophylactic antifungal therapy was required. Our patient also encountered oral and vaginal ulcers that appeared of non-infectious nature and were assumed to represent autoimmune manifestations related to *STAT1* GOF mutation (Figure 1). Treatment with steroids led to rapid improvement of these ulcers, but steroid sparing agents including azathioprine, hydroxychloroquine, and mycophenolate mofetil, were only of minor benefit. Upon treatment with anti-tumor necrosis factor (TNF)-α, adalimumab, (in combination

with antifungal therapy) ulcers did not reoccur.

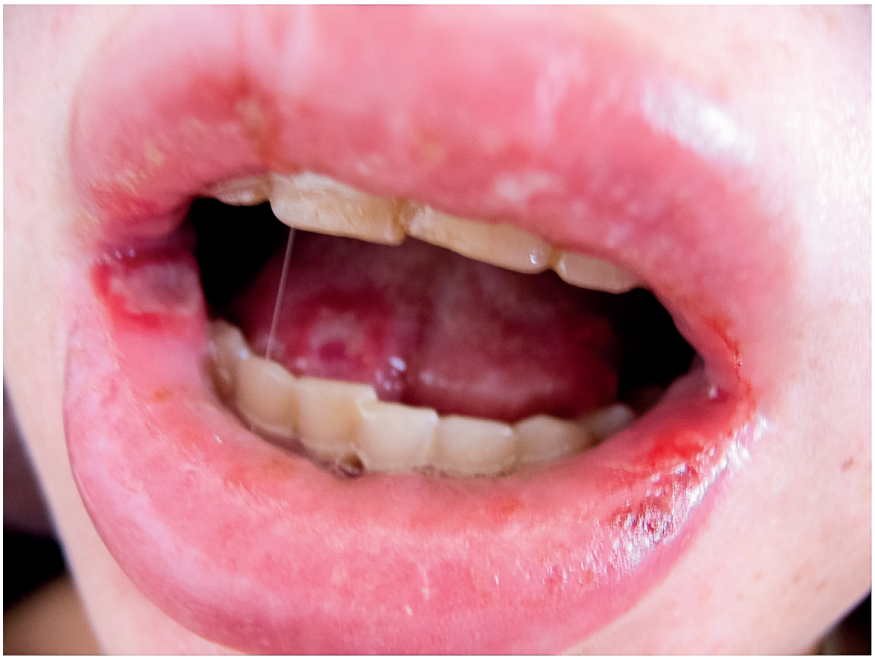

Figure 1. Recurrent oral ulcers in the patient. Multiple shallow ulcers with erythematous borders were found at upper lip, lower lip and tongue.

To demonstrate an evidence-based rationale to initiate baricitinib treatment, we first examined effectiveness of baricitinib *in vitro* using fresh blood from the patient and one age-gender-race-matched healthy control. The concentrations of baricitinib used in this study were based on serum levels achieved in patients on oral baricitinib 2-20 mg daily[8]. T lymphocytes from the patient displayed higher STAT1 phosphorylation levels when stimulated with IFN-α, IFN-β or IFN-γ than T lymphocytes from healthy control. IFNs induced phosphorylated STAT1 (pSTAT1) levels in the patient were decreased upon addition of baricitinib (Figure 2A). STAT3 phosphorylation, which is crucial for Th17 development[9], was also measured. Baricitinib enhanced IL-6-induced phosphorylated STAT3 (pSTAT3) level but had no effect on IFN-α-induced pSTAT3 in T lymphocytes from the patient (Figure 2B). Baricitinib at a concentration of 20 nM significantly enhanced heat-killed *Candida albicans* (HKCA) induced IL-17A production by peripheral blood mononuclear cells (PBMC) from the patient. However, this was not observed for higher concen-

trations of baricitinib (100 nM and 500 nM), suggesting an immunomodulatory effect within a specific dose range (Figure 2C). Baricitinib did not enhance IL-17F or IL-22 production (data not shown). In long-term T lymphocyte cultures, baricitinib significantly reduced expression of the STAT1 regulated genes *CXCL9*, *CXCL10*, and *CD274* (PD-L1) after IL-27 stimulation (Figure 2D).

Based on the *in vitro* data, baricitinib treatment (2 mg once daily) was initiated in the patient after adalimumab treatment had been terminated for 2 weeks. At the start of baricitinib treatment, no active candidiasis or ulcers were reported. Prophylactic antifungal therapy (fluconazole 200 mg once daily) was initially continued. As clinical condition remained stable, dose of fluconazole was reduced and fully tapered after three months of baricitinib. During 6 months of follow-up, no oral or vaginal ulcers reoccurred. Mucocutaneous candidiasis did not reappear even without prophylactic antifungal therapy. No clinical or biochemical complications were reported.

Blood samples collected before start of baricitinib and every 2 – 4 weeks afterwards, were examined for STAT1/STAT3 phosphorylation, STAT1-regulated gene expression and cytokine production. Before treatment, pSTAT1 in T lymphocytes from the patient was higher than pSTAT1 in five age-gender-race-matched healthy controls, as reported previously[4]. Baricitinib treatment reduced pSTAT1 levels, at every time point examined (Figure 3A). STAT3 phosphorylation also declined upon baricitinib treatment (Figure 3B). The remarkably high expression levels of STAT1-regulated genes (*CXCL9* and *CXCL10*) from patient PBMCs before baricitinib treatment were strongly decreased upon treatment (Figure 3C). Patient PBMCs obtained during baricitinib treatment displayed higher production of IL-17A, IL-17F, and IL-22 upon stimulation with phorbol 12-myristate 13-acetate and ionomycin (PMA+I) or HKCA than PBMCs obtained before treatment. However, these levels were much lower than those observed in healthy controls (n=5) (Figure 3D).

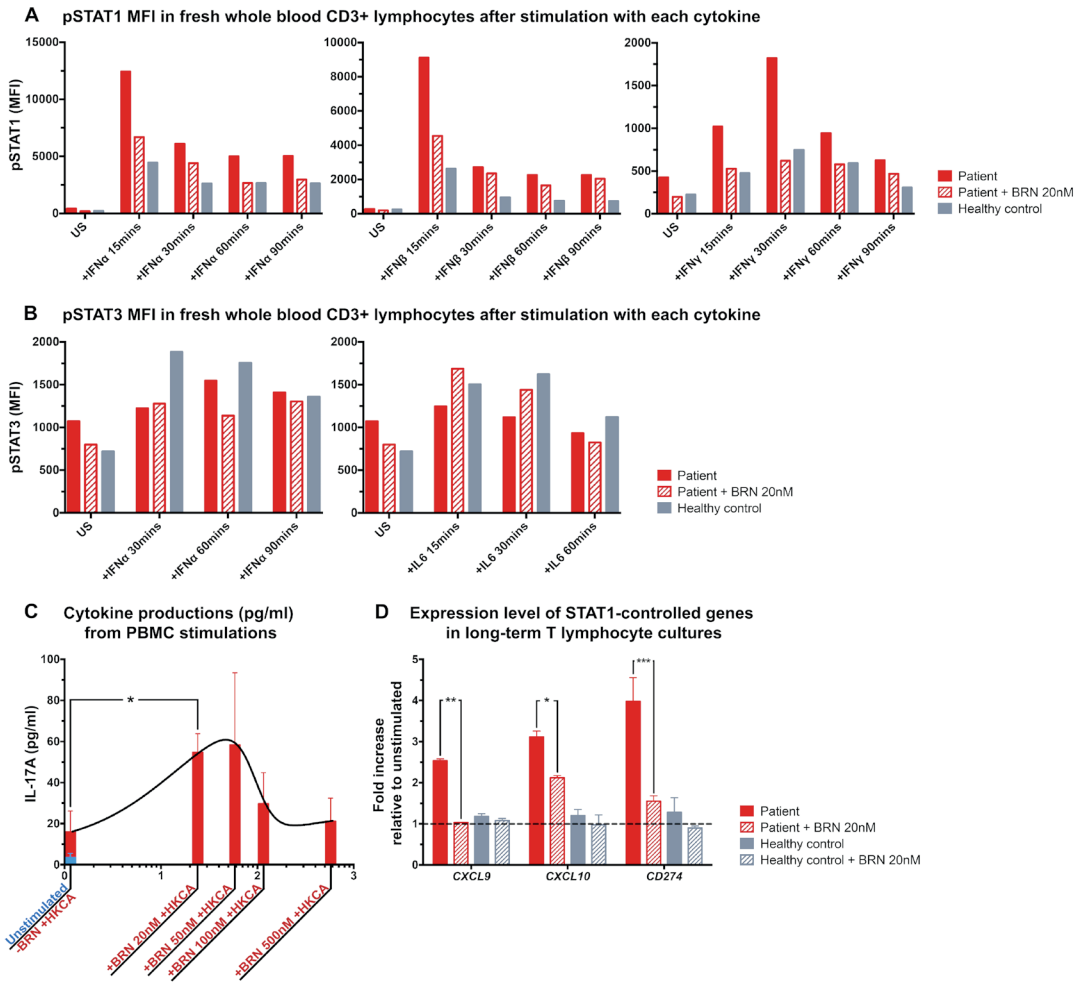

Figure 2. In vitro baricitinib efficacy studies. **(A-B)** Kinetics of STAT1 phosphorylation (A) and STAT3 phosphorylation (B) in fresh whole blood CD3+ T lymphocytes upon stimulation with IFN-α, IFN-β, IFN-γ, or IL-6 for indicated time periods. Samples were pre-incubated with baricitinib for one hour before stimulation. **(C)** Bar graph depicting IL-17A production in culture supernatant from patient PBMC after five days of stimulation with HKCA. Samples were pre-incubated with baricitinib for one hour before stimulation. **(D)** Long term T lymphocyte cultures were established, pre-incubated with baricitinib for one hour, and stimulated for 24 hours with IL-27. mRNA expression levels of the STAT1 regulated genes *CXCL9*, *CXCL10*, and *CD274* were determined by real-time quantitative Taqman PCR (qRT-PCR) and normalized to the housekeeping gene GAPDH. (C, D: data depicted are mean of three replicate experiments, error bars indicate standard error of the mean (SEM), US = unstimulated, HKCA = heat-killed Candida albicans, BRN = baricitinib, *P < .05, **P < .01, ***P < .001.)

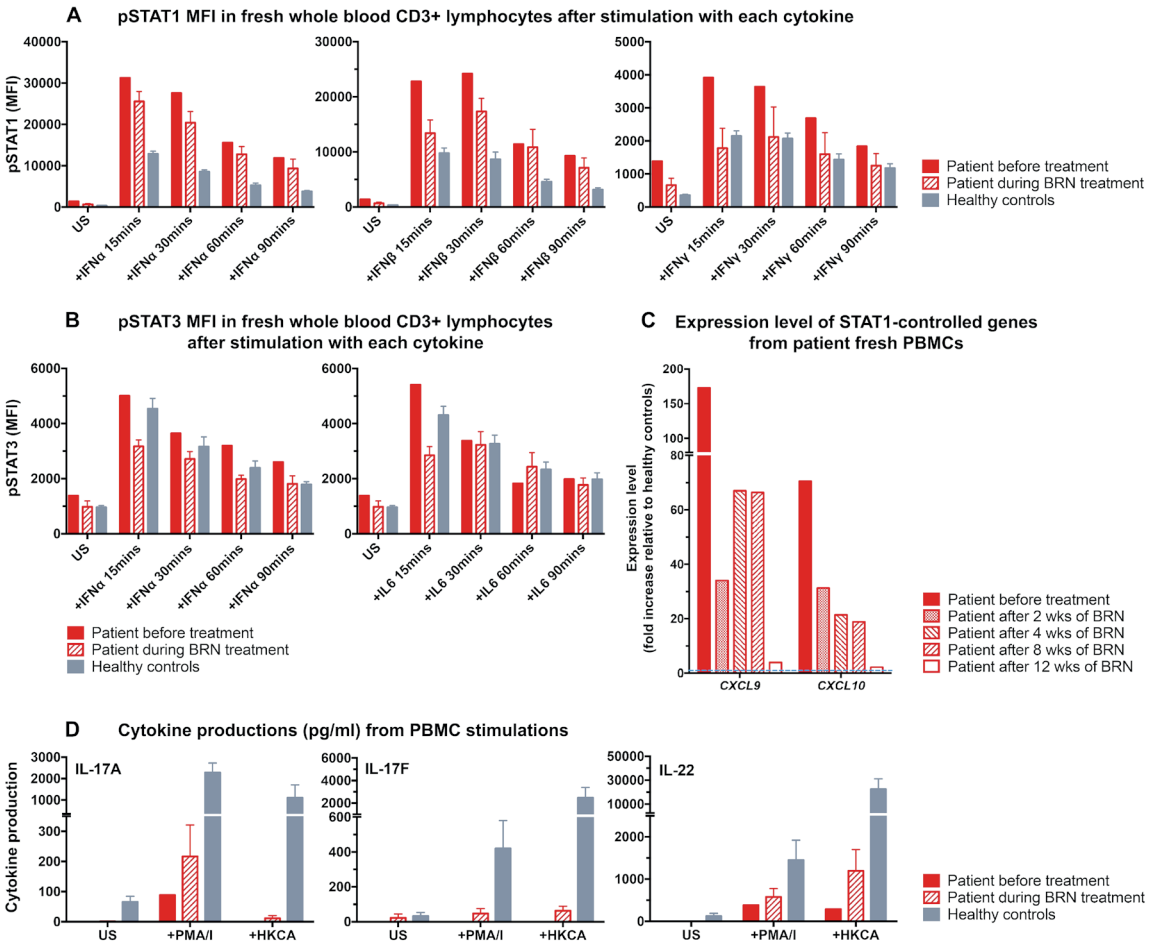

Figure 3. Immunological responses of peripheral blood cells obtained from the patient before and during baricitinib treatment. Blood samples were collected just before baricitinib was started and after 2 weeks, 4 weeks, 8 weeks, and 12 weeks of treatment. **(A-B)** Kinetics of STAT1 phosphorylation (A) and STAT3 phosphorylation (B) in fresh whole blood CD3+ T lymphocytes upon stimulation with IFN-α, IFN-β, IFN-γ, or IL-6 for indicated time periods. Enhanced pSTAT1 level in the patient observed before baricitinib treatment (solid black line) was decreased after treatment (dashed black line, depicts the average MFI from the four time-points of blood sampling post baricitinib initiation as samples from all time-points showed comparable results). Inducible pSTAT3 in the patient also decreased under baricitinib treatment (gray lines in A and B indicate average MFI from healthy controls (n=5)).

Figure 3. (continued) (C) Bar graph depicting mRNA expression levels of CXCL9 and CXCL10 in patient PBMC determined by qRT-PCR. Data were normalized to the housekeeping gene GAPDH. **(D)** Patient and healthy control (n=5) PBMCs were stimulated with PMA+I or HKCA. IL-17A, IL-17F, and IL-22 production was measured (ELISA) in culture supernatants after five days of culture. US, unstimulated, PMA + I = phorbol 12-myristate 13-acetate and ionomycin, HKCA = heat-killed *Candida albicans*. All the results with error bars indicate average value with standard error of mean (SEM).

This study provides the first evidence that baricitinib could be of value in the treatment of patients with *STAT1* GOF mutation. We demonstrate this at several levels. Firstly, our patient showed remarkable clinical improvement upon baricitinib treatment. Systemic prophylactic antifungal therapy and immunosuppressive treatment could be terminated, without reoccurrence of candidiasis or ulcers. Secondly, baricitinib reduced STAT1 hyperphosphorylation and STAT3 phosphorylation, and improved the capacity of PBMCs to produce IL-17A, IL-17F, and IL-22, cytokines crucial for antifungal immune responses. Thirdly, baricitinib reduced expression of the IL-27/STAT-1 regulated genes *CXCL9*, *CXCL10*, and *CD274* in long-term T lymphocyte cultures from the patient. PBMCs obtained from the patient during three months of baricitinib therapy also expressed *CXCL9* and *CXCL10* at levels approaching those of healthy controls. Overexpression of PD-L1 in naïve T lymphocytes from patients with *STAT1* GOF mutation is associated with enhanced IL-27/STAT1 signaling and contributes to inhibition of Th17 differentiation[2,3]. An anti-human PD-L1 inhibitory antibody was found to partially rescue IL-17A production in T lymphocytes from patients with *STAT1* GOF mutation[2]. Therefore, reduction of PD-L1 upon baricitinib treatment could contribute to the partial restoration of IL-17A production. In conclusion, we show for the first-time therapeutic benefit of the clinically available baricitinib in a patient with GOF *STAT1*. Further studies are required to evaluate its clinical implications in other patients with *STAT1* GOF mutation.

Methods

STAT phosphorylation analysis

Baricitinib (Selleck Chemicals, Houston, USA) was added to cells one hour prior to the stimulation. Stimulations with IFN-α (10^4 IU/ml; PeproTech, London, UK), IFN-β (10^3 IU/ml; tebu-bio, Le-Perray-en-Yvelines, France), IFN-γ (10^5 IU/ml; R&D systems, Abingdon, UK), or IL-6 (100 ng/ml; R&D systems) were performed for various durations and as previously described [4]. For flow cytometry analysis,

cells were fixed and permeabilized with permeabilizing reagent (Phospho-Epitopes Exposure kit; Beckman Coulter). Cells were stained with APC-conjugated antihuman CD3 (BD Biosciences, CA, USA), Fluor® 488-conjugated phospho-STAT1 Tyr701 (Cell Signaling Technology, MA, USA) and PE- conjugated phospho-STAT3 Tyr705 (Cell Signaling Technology, MA, USA) antibodies.

Cytokine production

PBMCs were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland), containing 10% heat inactivated fetal calf serum and penicillin and streptomycin (Cambrex BioWhittaker, Verviers, Belgium). Baricitinib was added to the cells for one hour prior to stimulation with phorbol 12-myristate 13-acetate (PMA) (81 nM) and ionomycin (I) (1.3 μM; eBioscience, CA, USA) or heat-killed *Candida albicans* (HKCA: 10^6 cells). After five days of culturing, supernatant was collected and analyzed for IL-17A, IL-17F, and IL-22 with enzyme-linked immunosorbent assay (ELISA; R&D systems).

Long-term T lymphocyte culture

Long-term T lymphocyte cultures were established as previously described [4]. After two weeks of culturing, T lymphocyte cultures with a purity of > 90% were obtained. Baricitinib was added to the cells for one hour prior to stimulation with IL-27 (200 ng/ml; R&D systems) for 24 hours. Subsequently, cells were collected and analyzed for *CXCL9*, *CXCL10*, and *CD274* gene expression with real-time PCR.

Real-time PCR

Total RNA was extracted from cultured cells with GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, MO, USA) according to manufacturer's protocol. RNA was reverse transcribed into cDNA with random primers (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). PCR for *CXCL9*, *CXCL10*, and *CD274* was performed using primer probe mix (Thermo Fisher Scientific) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene expression data were normalized to the expression of the housekeeping gene *GAPDH*.

Statistical analysis

Statistical analysis for cytokine production experiment was performed with one-way ANOVA with Bonferroni's multiple comparison test and gene expression experiment with t-test. All assessment was conducted with GraphPad Prism (GraphPad Software; San Diego, Calif software). Statistical significance was considered when $P < .05$ in all analyses.

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4

INTERFERONOPATHY

Baricitinib Treatment in Aicardi-Goutières Syndrome

CHAPTER 4

Baricitinib Treatment in Aicardi-Goutières Syndrome

Efficacy of Baricitinib in the Treatment of Chilblains Associated with the Type I Interferonopathy Aicardi-Goutières Syndrome

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Arthritis Rheumatol. 2019 May; 71(5): 829–831

To the Editor:

Aicardi-Goutières syndrome (AGS) is a rare early-onset auto-inflammatory disease characterized by encephalopathy, basal ganglia calcification, chronic cerebrospinal fluid (CSF) lymphocytosis, and elevated type I interferon (IFN) levels in the CSF[1,2]. Typical clinical manifestations include intellectual impairment, dystonia, and seizures. AGS patients usually experience active disease during the neonatal or infancy period, after which disease activity attenuates and subsequently stabilizes[1,3,4]. Apart from neurological symptoms, some patients also develop digital vasculitis or necrosis of the skin (chilblain), panniculitis, glaucoma, and other clinical signs that are also observed in systemic lupus erythematosus (SLE), including thrombocytopenia, arthritis, and antinuclear antibody (ANA) positivity[5,6-8].

AGS is classified as a monogenetic type I interferonopathy with autoinflammation resulting from constitutive upregulation of type I IFN signaling[5]. IFN-stimulated genes (ISGs) are constantly overexpressed in peripheral blood cells from AGS patients and measurement of type I IFN signature in these cells represents a useful marker for AGS disease activity[9,10]. At least seven distinct gene mutations were reported, including mutations in *SAMHD1*[1,3]. Loss-of-function mutations in *SAMHD1* lead to reduced SAMHD1 enzyme activity, which is crucial for cytosolic deoxynucleotide (dNTP) metabolism[1,2]. The defective pathway of cytosolic dNTP induces type I IFN production via cyclic GMP–AMP synthase (cGAS), stimulator of IFN genes (STING), and interferon regulatory factor 9 (IRF9)[1,2].

Janus kinase/signal transduction and activator of transcription (JAK/STAT) activation is crucial in the signaling cascade of numerous cytokines involved in the pathogenesis of various autoimmune diseases[11]. Consequently, treatment with specific JAK inhibitors including ruxolitinib and tofacitinib in immune-mediated diseases has been increasingly reported[12,13,14]. Recently, the oral JAK 1/2 inhibitor baricitinib was approved for the treatment of active rheumatoid arthritis and was also found to be effective in the treatment of a patient with chronic mucocutaneous candidiasis due to a *STAT1* gain-of-function mutation[15,16]. Therefore, baricitinib is of potential interest to modulate the effects of IFN (over)exposure in patients with a type I interferonopathy. In this report, we describe an AGS patient treated with baricitinib and demonstrate its potential clinical applications for treatment of type I interferonopathies.

Our currently 22-year old female Caucasian patient with a consanguineous family history was diagnosed with AGS at the age of 19 years based on a homozygous nonsense mutation in exon 4 of *SAMHD1* (c.490C>T, p.(Arg164Ter)). The identified mutation has been described previously for AGS5[17] and was classified as a disease-causing mutation in the Human Gene Mutation Database (HGMD). Her medical history includes subclinical hypothyroidism, short stature, and mild intellectual disability. Calcifications of the basal ganglia were found on computed tomography scan in 2007. The most prominent clinical feature is severe chilblains which have been active over many years. Scaly and crusted ulcers from chilblains were constantly observed on both hands and feet (Figure 1). The inflammation and pain typically exacerbated after cold exposure.

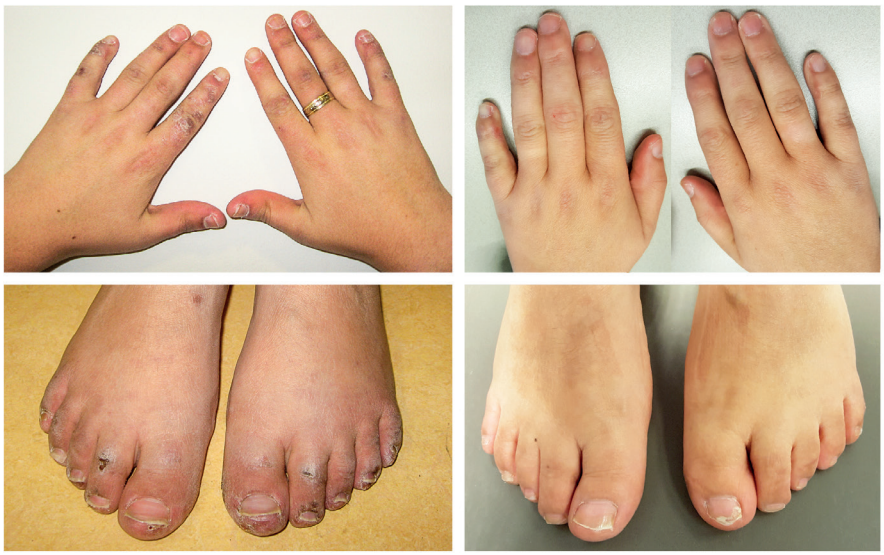

Figure 1. Clinical response of AGS patient to treatment with baricitinib. Bilateral ill-defined scaly and erythematous patches with some minute crusted ulcers from chilblains were found on both hands and feet before treatment (left). Clinical improvement during 5th month of treatment (right)

Baricitinib treatment was initiated at a daily dose of 2 mg. At the start of baricitinib treatment, the patient experienced active chilblains on both hands and feet. After 6 weeks of treatment, the lesions completely resolved. Until now, after 15 months of treatment, no recurrence of chilblains was described (Figure 1). The lesions also did not reappear during winter, when the disease was usually active. Although (re)occurrence of viral infections and other opportunistic infections has been reported on treatment with JAK inhibitors in autoimmune disease[18], these were not encountered by our patient. Moreover, no other clinical or biochemical complications were reported.

Peripheral blood samples were collected from this patient 4 weeks prior to the start of baricitinib and after 2 and 6 weeks of treatment. Expression levels of five ISGs (*IFI44*, *IFI44L*, *IFIT3*, *LY6E* and *MX1*), that represent the gene signature for type I IFN activity[19], were measured in isolated CD14+ monocytes and compared to the expression level of 54 healthy controls. Prior to baricitinib treatment, monocytes from the patient displayed higher expression of all tested ISGs compared to the healthy controls (Figure 2A). Expression of all five ISGs remarkably declined upon baricitinib treatment (Figure 2A). This data indicates that the type I IFN gene signature is associated with disease activity in AGS and represents a reactive biomarker in the context of effective treatment.

Furthermore, we measured total STAT1 and phosphorylated STAT1 (pSTAT1) in peripheral blood T lymphocytes by flow cytometry[16]. T lymphocytes from the patient before and during treatment expressed higher levels of total STAT1 (Figure 2B) than that observed in T lymphocytes from two age-gender-race matched healthy controls. T lymphocytes from the patient before and during baricitinib displayed baseline levels of pSTAT1 comparable to that of the healthy controls (Figure 2C-D). However, T lymphocytes obtained from the patient before initiation of baricitinib treatment displayed far higher levels of pSTAT1 upon IFN-α stimulation than T lymphocytes from the healthy controls (Figure 2C-D). T lymphocytes from the patient that were obtained during the period of baricitinib treatment displayed a strong reduction in pSTAT1 upon IFN-α stimulation in comparison to that observed in the T lymphocytes obtained before baricitinib treatment (Figure 2C-D).

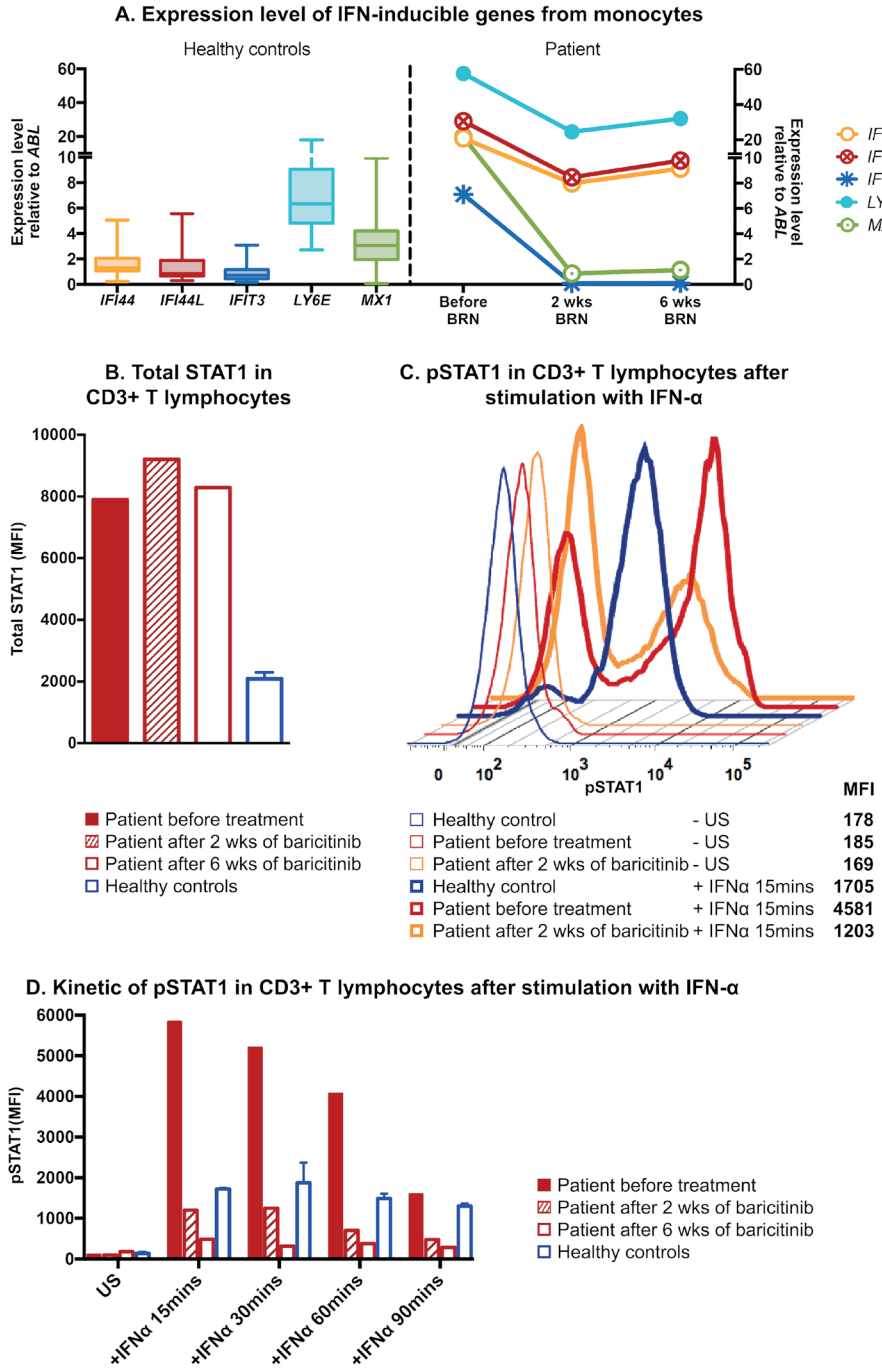

Figure 2. Immunological responses of peripheral blood cells obtained from the patient before and during baricitinib treatment. Blood samples were collected before baricitinib was started and after 2 weeks and 6 weeks of treatment.

Figure 2. (continued) (A) Box & Whisker plot (left) depicting mRNA expression levels of the IFN-stimulated genes (ISGs) *IFI44*, *IFI44L*, *IFIT3*, *LY6E* and *MX1* in monocytes from healthy controls (n=54). Line graph (right) depicting mRNA expression levels of the ISGs in monocytes from the patient before and during the treatment. The expression levels were determined by qRT-PCR and the data were normalized to the housekeeping gene ABL. **(B)** Bar graph showing total STAT1 levels in T lymphocytes from the patient before and during treatment as well as in healthy controls. **(C)** Histograms showing STAT1 phosphorylation in T lymphocytes upon type I IFN induction. **(D)** Bar graphs depicting kinetics of STAT1 phosphorylation in T lymphocytes upon stimulation with IFN-α for the indicated time periods. MFI = mean fluorescence intensity, wks = weeks, BRN = baricitinib, mins = minutes, US = unstimulated. All the results with error bars indicate average value with standard error of mean (SEM)

This study demonstrates that baricitinib could be beneficial in the treatment of patients with AGS. This is supported by several observations. First, clinical improvement of the patient was found, as the chilblain lesions were completely healed after 6 weeks of treatment, without reoccurrence for over 15 months. Secondly, the high expression level of ISGs in monocytes from the patient declined during the treatment with baricitinib. Thirdly, despite the high total STAT1 level in the patients T lymphocytes, baricitinib treatment prevented STAT1 hyperphosphorylation in these cells when stimulated with IFN-a.

In summary, we present for the first time that baricitinib is a novel drug for the treatment of chilblains in AGS patients with a *SAMHD1* mutation and consequent upregulation of type I IFN activity. The immunological effects of JAK-inhibitors depend on their selectivity and inhibitory capacity for the several JAK subtypes. Baricitinib displays a stronger inhibitory effect on cytokine induced STAT phosphorylation than ruxolitinib which was previously reported successful in the treatment of STING-associated type I Interferonopathy[13,20]. Therefore, more in-depth research is warranted to evaluate clinical response to baricitinib treatment for type I interferonopathies.

Acknowledgement
We are grateful to Prof Yanick Crow of Manchester Center for Genomic Medicine, Saint Mary's Hospital, Manchester for sharing his idea that JAK inhibitor might work for the patient's chilblains.

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5

DISCUSSION

Pathophysiology of Genetic variants in STAT1 and STAT3 Associated with Immune Dysregulation in Primary Immunodeficiency Disorders

Interactions between STATs in the Pathophysiology of PIDs with Immune Dysregulation

JAK/STAT Pathways as Target for Therapy

PATHOPHYSIOLOGY OF GENETIC VARIANTS IN *STAT1* AND *STAT3* ASSOCIATED WITH IMMUNE DYSREGULATION IN PRIMARY IMMUNODEFICIENCY DISORDERS

Pathophysiology of *STAT1* GOF variants

JAK/STAT signaling pathways are involved in several immunological and non-immunological responses. Both germline loss-of-function (LOF) and gain-of-function (GOF) variants in *STAT*1 have been reported, and these mutations are closely related to primary immunodeficiency disorders (PIDs). While LOF variants in *STAT1* predominantly result in immunodeficiency with increased risk for viral and/or mycobacterial infections, GOF variants in *STAT1* lead to a clinical phenotype involving both immunodeficiency and autoimmunity[1]. Patients with *STAT1* GOF variants are also at increased risk to develop vascular aneurysms and cancer when compared to the general population[1]. GOF variants in *STAT1* are highly associated with enhancement of STAT1 phosphorylation. The increased phosphorylation pattern is determined by the STAT1 domain containing the particular variant. Activating variants in the coiled-coil domain (CCD) and DNA binding domain (DBD) of the STAT1 protein lead to increased and prolonged STAT1 phosphorylation. Variants located in SH2 domain, as also shown by the studies in Chapter 2.1, lead to increased STAT1 phosphorylation, without prolonged phosphorylation[2-5]. Moreover, GOF variants in *STAT1* may also result in diminished Th17 responses in terms of impaired IL-17A, IL-17F, and IL-22 production and/or reduction of Th17 lymphocyte numbers[3,4,6]. The disturbed Th17 function is proposed to be the major cause of CMC in patients with GOF variants in STAT1. The study presented in Chapter 2.1 also detected higher expression of STAT1-targeted genes, including CD274 (PD-L1) in T lymphocyte cultures carrying *STAT1* GOF variant. Increased expression of PD-L1 was found in T lymphocytes of the patients with *STAT1* GOF variants and is associated with impairment of Th17 lineage development[7,8]. Although STAT3 is a crucial transcription factor for Th17 development, STAT3 phosphorylation is usually unaffected in cells with the *STAT1* GOF variants[6].

The pathophysiology of autoimmunity in these patients still needs to be explored. Notably, while CMC is also reported in the patients with LOF inborn errors of IL-17A/F immunity and in patients with AD-HIES, autoimmune phenotypes are not common in these patients. Various phenomena of immune dysregulation in patients with *STAT1* GOF variants resemble patients with IPEX syndrome or

APECED. However, GOF variants in *STAT1* do not disturb either IL-2-mediated STAT5 phosphorylation, FOXP3 expression, or (regulatory T lymphocyte) Treg development, while these immunological phenotypes are described in IPEX syndrome[9]. Patients with LOF variants in *AIRE* also display clinical overlap with *STAT1* GOF patients including CMC, autoimmune thyroiditis, alopecia, and type 1 diabetes. Yet, a recent study reported that STAT1 expression in monocytes from APECED patients was lower than that of healthy controls which is in the opposite direction with *STAT1* activating variants[10]. Immune dysregulation in *STAT1* GOF variants might be due to an increased STAT1-mediated IFN-α/β signaling which is also the case in patients with interferonopathies.

Patients with *STAT1* GOF variants have a higher risk of developing aneurysms and vasculitis. Most of the vascular diseases in these patients occur in the central nervous system[1,11]. A direct role of STAT1 in aneurysm formation is not well established yet. Studies of the atherosclerosis process revealed that STAT1 mediates stimulation of aortic endothelial cell growth by the induction of vascular endothelial growth factor (VEGF) and angiotensin II[12]. Signaling of STAT1 through activation of TLR4 also results in expression of several pro-inflammatory and pro-atherogenic mediators, including CXCL10 and CCL5[13-15]. These evidences unveil the contribution of STAT1 in vascular remodeling and might explain vasculitis as well as aneurysm formation in *STAT1* activating mutations. On the other hand, a study also reported inhibition of IFN-γ-induced STAT1 signaling on angiogenesis through suppression of VEGF activity[16]. Therefore it can be speculated that vasculitis (with or without formation of aneurysms) in the patients with *STAT1* GOF mutation is caused by type I IFN-mediated STAT1 signaling since the incidence of vascular disease seems to be more frequently occurring in patients with autoimmune phenotypes, particularly type I diabetes[1]

Patients with *STAT1* GOF variants have an increased risk of developing cancer, particularly squamous cell carcinoma of the skin, oral cavity, larynx, and esophagus. In several types of cancer, STAT1 is known as inducer of anti-proliferative and pro-apoptotic genes that inhibit tumor growth[17]. STAT1 also generates cancer promoter effects in some specific circumstances. In general, the burden of inflammation in inflammatory diseases is associated with development of malignancies. In *STAT1* GOF mutations, the development of carcinoma is related to candidiasis[18]. Candida infection is known to increase the risk of tumor progression[19]. Although *C. albicans* is a commensal yeast of the human body, during infection, *C. albicans* produces

carcinogenic compounds including nitrosamine and acetaldehyde[20]. Nitrosamine is able to induce cellular dysplastic changes and provoke malignant tumors in various tissues[20,21]. Acetaldehyde is also related to formation of carcinogenic metabolites that may interfere with DNA replication and DNA repair machinery[20,22].

Pathophysiology of *STAT3* GOF variants

In several studies, the function of STAT3 has been unveiled as promotor of cell survival and development of autoimmune diseases. In line with *STAT1* variants, LOF variants in *STAT3* result in AD-HIES with a clinical picture dominated by an increased risk of bacterial infections and CMC without a distinct autoimmune phenotype[23]. GOF variants in *STAT3* lead to immune dysregulation disorders including immunodeficiency and autoimmunity[24]. To better understand the pathophysiology of each clinical symptom of patients with *STAT3* GOF variants, additional in-depth studies and reported case series are required as the clinical phenotype of the disease is highly diverse. Patients with *STAT3* GOF variants are susceptible to virtually all micro-organisms, with variable degrees of severity. Immunodeficiency in patients with *STAT3* activating variants varies from hypogammaglobulinemia to aberrancy of total immune cell numbers and/or immune cell functions[25-27].

STAT3 plays an important role in the induction of inflammatory responses, and as expected, GOF variants in *STAT3* result in multiple early onset autoimmune features[24]. However, the mechanism of autoimmunity is not always straightforward as the immunological phenotypes in these patients are diverse. Increase in Th17 and/or reduction of Treg responses are mostly observed[25,26]. Yet, a number of patients experience autoimmune phenomena with normal Th17 and/or normal Treg responses[26,27]. Additionally, as STAT3 promotes cell proliferation, somatic GOF variants in *STAT3* are also associated with T cell and NK cell large granular lymphocyte leukemia[28,29]. STAT3 may thus act as a malignancy driving transcription factor. Therefore, patients with germline *STAT3* GOF variants may be also at risk for cancer. However, so far, only few patients with germline *STAT3* activating variants have been described with large granular lymphocytic leukemia or Hodgkin lymphoma. Since most of the reported patients with these genetic variants are young, the risk of development of malignancy could increase over time and the treating physicians should be aware of these possible complications.

Chapter 3 of this thesis describes two patients with a novel Y360C *STAT3* GOF variant who developed severe pulmonary hypertension. Pulmonary

hypertension can develop as a consequence of pulmonary artery hypertension (PAH) or as a complication (secondary pulmonary hypertension), of other conditions, for example interstitial lung disease and cardiac conditions[30,31]. In our patients with *STAT3* GOF variants, pulmonary hypertension could be a complication of interstitial lung disease. However, several studies also described a role for STAT3 in development of PAH. Therefore, it is also possible that activating variants in *STAT3* itself directly results in PAH and pulmonary hypertension. STAT3 participates in the pathophysiology of PAH by initiating and promoting vascular smooth muscle cell (VSMC) proliferation and reducing of apoptosis of these cells[32,33]. Subsequent overgrowth of these endothelial cells generates a 'low-flow-high-resistance' system from vascular constriction, elevation of pulmonary vascular resistance, and elevation of pulmonary pressure[32,33]. Binding of STAT3 to the endothelial nitric oxide synthase (eNOS) promoter diminishes the promotor activity and leads to reduction of eNOS protein level. Depletion of eNOS protein results in insufficiency of nitric oxide which is an endothelium-derived vasorelaxant compound as well as an inhibitor of SMC growth[33,34]. Additionally, STAT3 activation upregulates nuclear factor of activated T cells (NFAT) and provirus integration site for Moloney murine leukemia virus (Pim1)[32,33]. Overexpression of these two transcription factors increases VSMCs proliferation[32]. Interestingly, pulmonary artery endothelial cells from patients with idiopathic PAH also expressed higher level of phosphorylated STAT3 than that of healthy controls[33,35]. Endothelial cells from patients with idiopathic PAH showed greater response to growth factors (e.g. VEGF, basic fibroblast growth factor [bFGF], human epidermal growth factor [hEGF], and IGF-I) as displayed by excessive proliferation rates, migration rates, and survival rate. This suggests that the IL-6/JAK/STAT3 pathway might be a target for therapy in PAH. Indeed, the pathological effects were strongly reduced by a JAK2/STAT3 inhibitor, AG490[35].

The effect of STAT3 on vascular disease could also be related to the retinal neovascularization in our patients with the L387R *STAT3* activating variant. STAT3 is also expressed in retinal pigmented epithelium (RPE) and is recognized as regulator of RPE survival, inflammation, cytokine production, and visual cycle maintenance[36]. Therefore, aberrancy in the STAT3 signaling pathway could contribute to both retinal vascular disease and macular edema which resulting in degeneration of the retina.

INTERACTIONS BETWEEN STATS IN THE PATHOPHYSIOLOGY OF PIDS WITH IMMUNE DYSREGULATION

Genes of the *STAT* family are proposed to originate evolutionary from only a single *STAT* gene that has undergone genome duplications into seven STAT members in humans[37]. Perhaps because of this proposition, all STATs can bind to the same palindromic DNA motif, GAS, yet with different affinity. In the equilibrium state of STATs, each STAT and STAT complex binds to its preferred GAS sites. However, reduction in number or function of one STAT protein allows other STATs to compensate for its GAS binding and function. Likewise, increase in number or function of one STAT also competes with other STAT family members GAS bindings and functions[38]. These phenomena could explain why the patients with *STAT1* GOF variants experience clinical symptoms that overlap with patients carrying *STAT3* LOF variants and the patients with *STAT3* GOF variants experience clinical symptoms that overlap with the patients carrying *STAT5B* LOF variants. Functional discrepancy between STAT homodimerizations and heterodimerizations might also explain the shared clinical phenotypes. As an example, the STAT1:STAT3 heterodimer might not bind to IL-17 encoding loci as effective as a STAT3 homodimer[18]. The complexity in pathophysiology of STAT mutations therefore likely results from similarity of the STATs protein structure while each STAT exerts diverse effects including opposite functions in biological relevant processes.

STAT1 GOF and *STAT3* LOF variants

Patients with *STAT1* GOF and *STAT3* LOF variants are highly vulnerable to candidiasis. Susceptibility to candida infections in AD-HIES patients is well-described from reduction of STAT3-mediated Th17 response. The pathogenesis of the increased risk of candida infections in AD-HIES could be the same for patients with *STAT1* GOF variants as both of these groups of patients display diminished Th17 counts and/or function and both experience infections mainly from the same organisms. IL-17 also plays an important role in the protection against bacterial, viral, and parasitic infections[39]. Therefore, apart from fungi, infections with other pathogens are also frequently observed in these patients. Lymphocytes from both of the patients with *STAT1* GOF and *STAT3* LOF variants displayed increased cytokine-induced STAT1 phosphorylation[40]. Additionally, STAT1-dependent PD-L1 upregulation is highly elevated in both *STAT1* GOF and *STAT3* LOF lymphocytes and is associated with a significant reduction of *SOCS3* expression[8,40]. STAT1-

dependent-upregulation of PD-L1 is associated with impairment of Th17 response[8,40]. In several cell types, an opposing role between STAT1 and STAT3 was reported and reduction of STAT3-dependent gene transcription is found in cells carrying *STAT1* GOF variant[40,41]. Additionally, formation of STAT1:STAT3 heterodimers was suggested to contribute to the shared clinical phenotypes between patients with either *STAT1* GOF or *STAT3* LOF variants. STAT1:STAT3 heterodimers are considered to be less transcriptionally active than STAT1:STAT1 and STAT3:STAT3 homodimers[18]. Therefore, in the case of *STAT1* GOF variants, ligand activation leads to relatively high STAT1 homodimer and STAT1:STAT3 heterodimer formations, while STAT3 homodimer are formed at relatively low level. This situation yields the same as for *STAT3* LOF variant in which STAT3 homodimer and STAT1:STAT3 heterodimer formation are comparatively lower than STAT1 homodimer formation. Another remarkable evidence for the opposing roles between STAT1 and STAT3 is shown in cells carrying *STAT3* GOF variant. Impairment of STAT1 phosphorylation was also detected in many cases with *STAT3* GOF variants. Enhancement of SOCS3 expression in cells with *STAT3* GOF variant is proposed to be a feedback mechanism underlying the reduction of STAT1 phosphorylation in these cells[26]. Decreased phosphorylation of STAT1 in the patients with *STAT3* GOF variants potentially relates to susceptibility to viral and/or mycobacterial infections in these patients as is also described in the patients with *STAT1* LOF variants.

Vascular abnormalities are also reported in patients with *STAT1* GOF variants and HIES. Aneurysms are frequently observed vascular complications in both conditions. Tortuosity and dilatation of arteries are also noted in HIES[42]. Although HIES is also caused by mutations in other genes apart from *STAT3* (e.g., *DOCK8*, *PGM3*, *CARD11, TYK2)*, vascular abnormalities are mainly detected in the patients carrying AD *STAT3* LOF mutation[42,43]. This suggests that vascular abnormalities could be related to decreased function of STAT3. The pathology of vascular anomalies in AD-HIES are hypotrophic arterial walls, elevated circumferential stress, and susceptibility to arterial dilation[44]. One research group suggested the relation of vascular abnormalities to a connective tissue defect as characterized in the inherited Ehlers-Danlos vascular-type syndrome, since the patients with AD-HIES also display joint hyperextensibility[44]. More data of thorough arterial vasculature and hemodynamic study in patients with *STAT1* GOF mutations are still required to find the correlation between vascular abnormality in *STAT1* GOF and *STAT3* LOF variants. Several studies suggested that aneurysms reported in *STAT1* GOF variants are the consequences of inflammation of the arterial wall. However,

for *STAT3* LOF variants, the levels of systemic inflammatory biomarkers do not correspond with the vascular abnormalities. Autoimmune phenotype is also not common in *STAT3*-LOF-related AD-HIES[44]. Vascular aneurysms in *STAT3* LOF variants are therefore not supposed to be the effect of inflammation. However, in case of ruptured aneurysm, murine experiments demonstrated increase in severity including rupture of aneurysm after inhibition of IL-6-mediated STAT3 signaling or IL-17A signaling pathway[44]. Therefore, susceptibility to aneurysm rupture in both groups of patients with *STAT1* GOF and *STAT3* LOF variants is suggested to be the result of an insufficient Th17 response.

STAT3 GOF and _STAT5B_ LOF mutations

Postnatal growth failure is one of the major phenotypes in *STAT3* GOF variants and low levels of IGF-I were measured in most of the patients tested[24]. Therefore, growth defects in these patients are supposed to result from disruption of the GH/IGF-I axis. Interestingly, this phenotype is also displayed in patients with *STAT5B* LOF variants, since functional impairment of STAT5B is associated with partial GH insensitivity[45]. Growth delay in *STAT3* GOF patients might be related to disrupted function of the GH/STAT5B pathway. In many cell types, STAT3 and STAT5B have different and opposing effects on gene expression[45,46]. There are four possible explanations for the opposing effects of STAT3 and STAT5B. Firstly, GH activates STAT1, STAT3, STAT5A, and STAT5B via JAK2. The activation of STAT5s upon GH regulates IGF-1, while activation of STAT1 and STAT3 results in STAT1:STAT3 heterodimerization and transcription of the proto-oncogene *FOS*[46]. Therefore, enhancement of STAT3 activation might lead to an increased formation of STAT1:STAT3 heterodimers which deviates GH signaling from activation of STAT5 as usual. In line with this, some experiments demonstrated reduction of STAT5B phosphorylation in cells with *STAT3* activating variants[25,45]. In NK cells of the patients with *STAT1* GOF variants, decreased STAT5 phosphorylation was also observed in one study which was restored after treating the cells with ruxolitinib[47]. Secondly, the functional reduction of STAT5B could be the effect of elevated expression of STAT3-regulated *SOCS3* which negatively mediates JAK/STAT pathway signaling. However, increased *SOCS3* expression was not always observed, for instance, normal *SOCS3* expression was detected in *STAT3* GOF mutation cases with short stature[48]. Thirdly, competition of STAT3 and STAT5B in binding at the same genetic loci is reported for the locus encoding IL-17[49]. This might suggest competitiveness between STAT3 and STAT5B regulating transcription of growth-

related genes as well. Lastly, the opposing function of STAT1 and STAT3 is also shown in *STAT1* GOF variant with reduction of the Th17 response as discussed above. The formation of STAT3:STAT5b heterodimer is also described. Perhaps an opposing role between STAT3 and STAT5 may occurred from nonfunctional STAT3/STAT5B heterodimer in the same manner as with STAT1/STAT3 heterodimer[45].

Both patients with *STAT3* GOF and *STAT5B* LOF variants display immune dysregulation disorders. The shared clinical phenotypes include immunodeficiency, type I diabetes, autoimmune cytopenia, eczema, enteropathy, and autoimmune thyroiditis. Most of the patients with *STAT3* GOF and *STAT5B* LOF variants show diminished numbers and/or function of Tregs in combination with reduced FOXP3 expression. Notably, autoimmune phenotypes in these patients are also similar to patients with IPEX syndrome. Dysfunction of Tregs is therefore suggested to be a cause of immune dysregulation in *STAT3* GOF and *STAT5B* LOF variants as it was also documented as a cause of immune dysregulation in the IPEX syndrome[50]. Autoimmune symptoms in patients with *STAT3* GOF variants are usually broader and more severe than in *STAT5B* LOF variants. This might be explained by an increased Th17 response reported in some patients with *STAT3* GOF variants. However, data of Th17 numbers and function in *STAT5B* LOF patients is required to link this correlation. Interestingly, STAT3 and STAT5 bind the same DNA motif of the *IL7A–IL17F* locus in a competitive manner[49]. Binding of STAT3 on *IL17* locus promotes Th17 development while binding of STAT5 reverses the action. Therefore, fate determination of Th17 represents the balance between engagement of STAT3 and STAT5 to the sites along the *IL7A–IL17F* locus[49]. Apart from the *IL17* locus, STAT3 and STAT5 interact in cancer development by engaging on the same *BCL6* locus. In breast cancer cells, STAT3 increases the expression of BCL6 while STAT5 reverses this action of STAT3 by displacing STAT3 from the shared binding site[51].

More than one-third of the patients with *STAT3* GOF variants develop interstitial lung disease (ILD)[24]. Lung fibrosis is one of the complications of ILD and is initiated by alveolar epithelial cell injury[52]. Injury of alveolar epithelial cells generates a profibrotic environment and promotes formation of fibroblastic foci containing activated and proliferating (myo)fibroblasts. Accumulating (myo) fibroblasts deposit large amounts of extracellular matrix thereby destroying the normal pulmonary architecture[53]. Of note, primary idiopathic pulmonary fibrosis is also one of the main concerns in patients with *STAT5B* LOF variants. Activation of STAT3 by TGF-β and IL-6 are the suggested mechanisms underlying development of

lung fibrosis[52]. Increased STAT3 phosphorylation is detected in lung fibroblasts and alveolar type II epithelial cells from patients with idiopathic pulmonary fibrosis[52,54]. However, the relation between STAT5 and lung fibrosis is still unexplained.

Interestingly, over 80% of the patients with HIES display abnormal facial appearances[55]. Hemihypertrophy, prominent forehead, deep-set eyes, mild prognathism, broad nasal bridge, and wide and fleshy nasal tip were noted[56]. Skin changes with rough and prominent pores are also observed. Some of these features are similar to acromegaly which is a disorder that results from excessive levels of GH and IGF-1. These observations are also suggestive of the opposite function of STAT3 and STAT5 on the GH axis.

Various cases of germline *STAT1*, *STAT3*, and *STAT5B* variants are reported. Both, LOF and GOF variants identified in these genes provide precious information for the study of STATs biology. Comparison of the clinical phenotypes between *STAT1* GOF and *STAT3* LOF variants as well as between *STAT3* GOF and *STAT5B* LOF variants illustrate the reciprocal function of STATs. Having more or less of any single STAT does not result in dysfunction of only one STAT but may disturb the equilibrium of the multi-STAT system. Keeping the balance is therefore indispensable for the healthy state of STATs.

JAK/STAT PATHWAYS AS TARGETS FOR THERAPY

So far, several immune dysregulations have been reported as a consequence of an aberration in STAT expression and/or function. Activation of certain STATs also participates in the development of malignancy. Therefore, it is indisputable to propose the JAK/STAT signaling pathway as one of the therapeutic targets in several diseases. Discovery of JAK inhibitors (JAKinibs) broadens the treatment options for several diseases, including hematologic cancers and immune-related inflammatory diseases. In this thesis, a JAKinib is proposed as a personalized medicine or precision medicine for patients with *STAT* GOF variants and the interferonopathy; Aicardi-Goutières syndrome (AGS).

There is no practical curative treatment for *STAT1* GOF variants. The only curative treatment identified is allogeneic hematopoietic stem cell transplantation (HSCT). However, the survival rate is less than fifty percent[57]. Majority of patients with *STAT1* GOF variants suffer from CMC, therefore prophylaxis antifungal therapy is the first line of treatment in almost every case[1]. Long-term use of antifungal

medication results in drug resistance which is found in almost half of the patients and thus second- or third-line of treatments are required[1]. In the *STAT1* GOF variant individuals who develop autoimmunity, immunosuppressive treatment is usually initiated. Treatment with infusion of either GM-CSF, G-CSF, or IFN-α are of minor benefit[1].

Administration of a JAKinib impedes activation of JAK molecules which occurs upstream of STAT activation[58]. This thesis reports a family with an inherited *STAT1* GOF variant in the SH2 domain (Chapter 2.1). Enhancement of STAT1 phosphorylation was identified as the consequence of the variant in *STAT1* and responsible for the clinical phenotype including both severe immunodeficiency and autoimmunity. Preceding studies reported the successful treatment of patients with *STAT1* GOF variants with the JAK1/2 inhibitor, ruxolitinib[59-61]. However, for the study conducted in this thesis (Chapter 2.2) baricitinib was selected based on its superior selectivity for JAK1/2 and its positive effects found in the clinical trials in rheumatoid arthritis and systemic lupus erythematosus[62-65]. The patient described in this thesis that was treated with baricitinib suffered from immunodeficiency, dominated by an increased risk of fungal infections, and a systemic lupus erythematosus-like autoimmune phenotype. Baricitinib was tested in the patient cells *in vitro* to prove for the therapeutic benefit and was evaluated for dose-response before the administration in the patient. Interestingly, administration of baricitinib resulted in reduction of STAT1 hyperphosphorylation and also phosphorylation of STAT3, yet in a lesser extent. Although STAT3 phosphorylation was impeded in some degree by baricitinib, the overall outcome led to the restoration of IL-17 immunity and improvement of antifungal immune response. During baricitinib treatment, the patient experienced remarkable improvement. CMC did not reappear, even without prophylactic antifungal therapy. Oral and vaginal ulcers also did not reoccur. These results demonstrated the therapeutic benefit of baricitinib in this patient with *STAT1* GOF variant and also supports the idea that the functional balance between STATs is necessary for a healthy immune system.

In Chapter 4 the treatment of a patient with AGS with baricitinib is demonstrated. Up-regulation of type I IFN signaling was described as a major pathophysiology of AGS and results in autoinflammation[66]. A previous study reported a benefit of ruxolitinib in patients with interferonopathy[67]. One female patient who had persistent severe chilblain lupus was included in the study described in chapter 4. The PBMCs of this patient displayed enhanced STAT1 phosphorylation

upon IFN-α stimulation. Treatment with baricitinib resulted in reduction of STAT1 hyperphosphorylation upon IFN-α activation as well as reduction of IFN-stimulated signature genes which was associated with remarkable clinical improvement. During the treatment, IFN-activated STAT1 phosphorylation was decreased to a level that was much lower than the level of healthy controls. Therefore, the treating physician should be aware of the vulnerability to viral or mycobacterial infections as an adverse reaction upon baricitinib administration. However, up to now (2 years), there are no signs of unusual infections or reoccurrence of chilblains.

The patients with a Y360C *STAT3* GOF variant that are described in this thesis (Chapter 3) were also treated with a JAK inhibitor. Two patients were treated with tofacitinib which is categorized as pan-JAK inhibitor. During tofacitinib administration, one of the patients was tested for serum STAT3-related cytokines, IL-6, IL-17A, and IL-17F. High pre-treatment levels of all measured cytokines were decreased during tofacitinib administration (Figure 1A-C). *In vitro* IL-6 production from PBMCs was increased upon PMA/ionomycin stimulation when compared to the level before the treatment. However, this IL-6 production level decreased again at the last time point of measurement (Figure 1D). Despite extensive drug interventions including tofacitinib, the clinical picture of pulmonary hypertension in one patient did not improve and led to death. Nevertheless, the other patient was treated with diuretics in combination with tofacitinib (serum cytokine and *in vitro* IL-6 measurements were not performed in this patient). Her clinical condition of pulmonary hypertension improved significantly and during tofacitinib treatment no progression of PAH was observed. This patient experienced a significant subjective overall clinical improvement upon tofacitinib treatment. Treatment failure in the patients with *STAT3* GOF variants treated with a JAKinib was previously reported with ruxolitinib[60]. The ineffectiveness of the treatment in reversing clinical symptoms was suggested to be due to late-stage of the disease when ruxolitinib was started[60]. This could also explain the treatment failure in one of our patient cases. Therefore, treatment with a JAK inhibitor may be more successful when the it is initiated in early stage of the disease. Yet, studies that determine the "window of opportunity" are required.

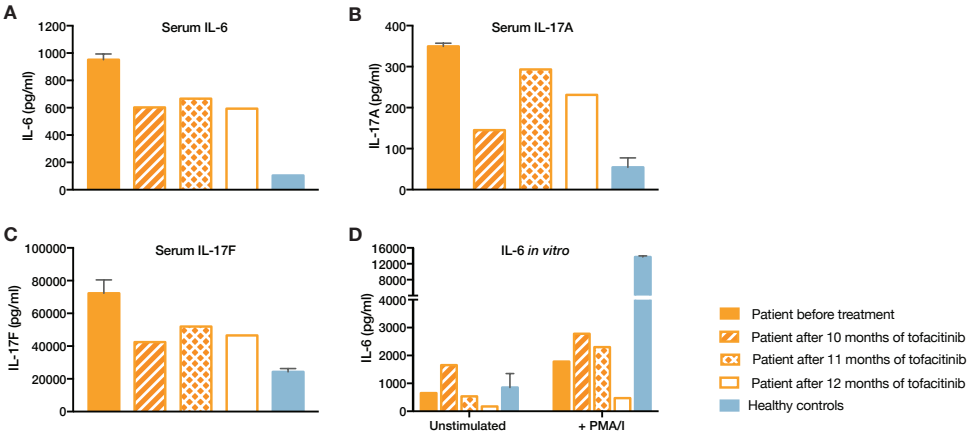

Figure 1. The effect of tofacitinib on cytokine production in the patient carrying *STAT3* p.Y360C GOF variant. **(A-C)** Serum level of IL-6, IL-17A, and IL-17F before and during tofacitinib administration. **(D)** *In vitro* IL-6 production by PBMCs stimulated with PMA/Ionomycin. Data with error bar are shown as mean ± SEM.

Based on clinical and immunological results, the treatment with a JAKinib in our cases, suggests a step forward in the direction of personalized medicine. In the diseases caused by hyperactivation of STATs, JAKinibs could precisely inhibit the targeted pathway which is expected to be better than unspecific immunosuppressive treatment or antimicrobial therapy. However, the challenge of inhibiting the STAT pathway in the treatment of immune-mediated diseases is that the dosage of JAKinib needs to be customized to the appropriated level, while in the treatment of cancer, a complete inhibition of the "target STAT" might necessary. Optimal dosage of JAKinibs treatment might also inhibit the other associated STAT pathways yet could lead to effective treatment from restoration of STATs equilibrium.

Discovery of highly specific STAT inhibitors may provide a more precise medication than JAKinibs for targeting the STAT pathway. However, inhibiting STAT is more difficult than inhibiting JAK activity due to similarity between each STAT structure. Additionally, inhibition of one specific STAT might generate a *STAT* LOF variant-like situation to the cells which may result in complications from imbalance of other STATs. Several compounds targeting SH2 domain or DBD of STATs have been developed but had unacceptable adverse reactions and bioavailability is also the major issue[63]. In the near future, restoring equilibrium of STATs might be approached by gene therapy especially for the diseases with a monogenetic mutation. CRISPR-Cas gene editing may be a promising future therapeutic approach

to recover the hematopoiesis. Several studies have demonstrated the possibility of using CRISPR-Cas systems in the human primary hematopoietic stem and progenitor cells for the treatment of hematological diseases including β-thalassemia and sickle cell disease[68]. Developing this technique for other monogenetic diseases could provide an effective, precise, and curative treatment for the patients.

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6

SUMMARY AND SAMENVATTING

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SUMMARY

The canonical janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway is a relatively simple but crucial membrane-to-nucleus pathway that orchestrates the human immune system. Variants in human *STATs* result in severe immune dysregulation including autoimmune disease and immunodeficiency. Treatment of patients with *STAT* variants is mostly symptomatic and there remains a need for additional effective therapies **(Chapter 1)**. Long-term use of antimicrobial therapies may lead to drug resistance and the effectiveness of several immune suppressive treatment modalities remains indefinite. Allogenic hematopoietic stem cell transplantation (HSCT) is the only curative treatment for patients with *STAT* variants, but has significant risk of secondary graft failure, infections and death. In this thesis, novel variants in *STAT1* and *STAT3* are presented. The studies were conducted to gain more insights in the biological and pathological effects of the described variants in *STAT1* and *STAT3*, which could provide a basis for precise pathway-targeted therapeutic strategies.

STAT1 gain-of-function (GOF) variants are identified as the underlying cause in about half of chronic mucocutaneous candidiasis (CMC) cases. Almost hundred heterozygous *STAT1* GOF variants have been described in the past decade. *STAT1* variants can be located in every domain of STAT1 but are mainly found in the coiled-coil domain and DNA binding domain. In **Chapter 2** of this thesis, a novel *STAT1* variant p.(V653I) is described in a Dutch family with four affected individuals. These patients suffer from CMC and various autoimmune phenomena. The identified variant is located in the SH2 domain of STAT1 and results in enhancement of STAT1 phosphorylation. In contrast to STAT1 variants located in other domains, an impaired STAT1 dephosphorylation rate was not observed. Expression levels of STAT1 regulated genes, *CXCL9*, *CXCL10*, and *CD274* (PDL1) in patient-derived long-term T lymphocyte cultures were higher than those in healthy controls. Cytokine-induced PD-L1 expression in T lymphocytes was previously found to diminish Th17 development. The V653I variant is also associated with impairment of patient PBMCs to produce IL-17A, IL-17F, and IL-22 which are crucial cytokines for human antifungal immunity. A diminished Th17 response is considered to be responsible for the clinical symptoms of CMC in the patients described in this study.

The immunological findings in the first study provided a basis for pathway

targeted therapy in these patients **(chapter 2.2)**. Based on the mechanism of action, a novel JAK1/2 inhibitor, baricitinib was proposed as a potential candidate. Prior to the treatment with baricitinib, the enrolled patient repetitively suffered from oral and esophageal candidiasis. Without systemic prophylactic antifungal therapy, she encountered candidiasis within two weeks. Our patients also experienced severe oral and vaginal ulcers which were assumed to represent autoimmunity related to the disease. After the treatment with baricitinib, the patient showed substantial clinical improvement. Prophylactic antifungal treatment and immunosuppressive therapy could be stopped without reappearance of candidiasis or ulcers. Baricitinib also decreased STAT1 hyperphosphorylation and increased IL-17A, IL-17F, and IL-22 production by the patient PBMCs. During treatment, expression levels of STAT1 targeted genes *CXCL9* and *CXCL10* were reduced, reaching levels comparable to those found in healthy controls. In this study we demonstrated, for the first time, the therapeutic efficacy of baricitinib in a patient with *STAT1* GOF variant on both immunodeficiency and autoimmunity.

We also demonstrated the benefit of baricitinib in a patient with Aicardi-Goutières syndrome (AGS) based on a *SAMHD1* variant in **Chapter 4**. The most prominent clinical feature was severe chilblains on hands and feet, which had been active over many years. Inflammation and pain were typically exacerbated after cold exposure. Within 6 weeks after initiation of baricitinib, the lesions completely resolved without reoccurrence for more than 2 years. The lesions also did not reoccur during winter. Five Interferon-stimulated genes (ISGs; *IFI44*, *IFI44L*, *IFIT3*, *LY6E*, and *MX1*) representing the gene signature for the activity of type I IFN were measured as disease markers. The elevated levels of ISGs, detected before initiation of baricitinib, remarkably declined during treatment. The hyperphosphorylation of STAT1 measured in T lymphocytes before baricitinib treatment also remarkably declined. Our findings suggest that baricitinib is a promising candidate for the treatment of chilblains in AGS patients with an upregulation of type I IFN activity.

Novel *STAT3* GOF variants are described in **Chapter 3** of this thesis. We discovered two new *STAT3* activating variants (p.[Y360C]) and p.[L387R]) in two families. From each family, two affected individuals were studied. Clinical signs and symptoms of these patients included immunodeficiency, autoimmune manifestations, dysmorphic features, and chronic lung disease. All of the patients had elevated circulating levels of STAT3 regulated cytokines, IL-6 and IL-17. *In vitro* studies using HEK293 cells transduced with heterozygous *STAT3* variants revealed

a defective nuclear translocation of the WT STAT3 allele, suggesting that these activating variants have a negative effect on the nuclear migration of the WT STAT3. These variants are also associated with increased STAT3 activity illustrated by luciferase reporter assays and the STAT3 downstream targeted gene, *SOCS3*, which is in line with the increased capacity to bind to target DNA. Non-canonical function of STAT3 in mitochondria was increased in the PBMCs carrying *STAT3* GOF variants. The oxygen consumption rate of patient PBMCs was significantly higher as compared to healthy controls. These variants are also associated with a reduction of naive T lymphocyte population and early exhaustion of T lymphocytes.

Two patients with the Y360C *STAT3* GOF variant and late-stage disease were treated with tofacitinib. In one patient, who suffered from advanced stage refractory pulmonary hypertension, no effect of tofacitinib was observed. In the other patient with the Y360C *STAT3* GOF variant, clinical condition improved upon treatment with tofacitinib. Pulmonary hypertension was well controlled, and no progression of disease was reported.

In **Chapter 5.1 and 5.2**, we discuss the biological effects of each member of the STAT family and their clinical consequences. The similarity between the various STAT structures, competition with the same target genes, and heterodimer formation are all suggested as mechanisms for the shared clinical phenotypes found among *STAT* loss-of-function (LOF) and GOF variants.

In **Chapter 5.3**, treatment approaches targeting the JAK/STAT signaling pathway are discussed. We describe the treatment strategies used in our studies and the clinical outcomes. We further discuss the challenging future of new approaches for a more precise inhibition of JAK/STAT pathways in complex immune dysregulation disorders.

SAMENVATTING

De kenmerkende "Janus Kinase" (JAK) - "Signaltransducer and Activator of transcription" (STAT) signaalroute is een relatief eenvoudige maar cruciale celmembraan-tot-celkern moleculaire verbindingsweg die een belangrijke functie heeft in het menselijke immuunsysteem. In het menselijk lichaam zijn 7 STAT moleculen geïdentificeerd. Mutaties in de menselijke STAT moleculen resulteren in een ernstige ontregeling van het immuunsysteem, deze leiden tot immuundeficiëntieziekten (afweerstoornissen) of auto-immuunziekten en soms een combinatie van beiden. Effectieve rationele therapieën voor patiënten met STAT-mutaties zijn momenteel nog niet uitontwikkeld **(hoofdstuk 1)**. Gebruik van antimicrobiële medicijnen kunnen op termijn leiden tot resistentie van micro-organismen tegen deze geneesmiddelen en in het geval van auto-immuniteit is de effectiviteit van de verschillende immuun-onderdrukkende therapieën beperkt. Hematopoietische stamceltransplantatie (HSCT) ofwel beenmergtransplantatie en gentherapie zijn momenteel de enige curatieve therapieën maar hebben een ernstig bijwerkingenpatroon en zijn bovendien beperkt beschikbaar.

In dit proefschrift worden nieuwe mutaties in STAT1 en STAT3 gepresenteerd. De beschreven studies werden verricht om meer inzicht te verkrijgen in de biologische en pathologische effecten van de mutaties en om in geval van ziekte aangrijpingspunten te identificeren voor een op de individuele patiënt gerichte therapie.

STAT1 gain-of-function (GOF) mutaties die een verhoogde STAT1 functie veroorzaken zijn voor ongeveer de helft verantwoordelijk voor chronische mucocutane candidiasis (CMC) bij patiënten. Bijna honderd heterozygote STAT1 GOF-mutaties zijn het afgelopen decennium gepubliceerd. De mutaties bevinden zich in elk domein van het STAT1 molecuul, maar worden voornamelijk gevonden in het regulatie domein en het DNA-bindend domein. In **hoofdstuk 2** van dit proefschrift wordt een nieuwe STAT1-mutatie p.(V653I) beschreven in een Nederlands gezin met vier aangedane individuen. Deze patiënten leden aan de combinatie van CMC en verschillende auto-immuunziekten. De gevonden mutatie bevindt zich in het SH2-domein van STAT1 en resulteert in STAT1-hyperfosforylering. Hyperfosforylering is een proces wat nodig is om STAT1 te activeren. In tegenstelling tot de mutaties die zich in andere domeinen bevinden, werd een verminderde STAT1-defosforylatie niet waargenomen. Expressie van de door

STAT1 gereguleerde genen, CXCL9, CXCL10 en CD274 (PDL1) in lange termijn T-lymfocytenkweken van de patiënt waren hoger dan die van de gezonde controles. Cytokine-geïnduceerde PD-L1-expressie door de T-lymfocyten bleek de belangrijke Th17-productie te verminderen. De V653I-mutatie wordt dan ook direct geassocieerd met een verminderde IL-17A, IL-17F en IL-22T productie door T-lymfocyten, dit zijn de cruciale cytokines die nodig zijn voor de afweer tegen schimmels. De verminderde Th17-respons in deze studie wordt dan ook beschouwd als een verklaring voor de klinische symptomen van CMC bij deze patiënten.

De immunologische bevindingen in de voorgaande studies gaven directe aanwijzingen om een gerichte therapie te starten bij deze patiënten **(hoofdstuk 2.2)**. Op basis van het werkingsmechanisme, werd de nieuwe JAK1/2-remmer baricitinib voorgesteld als een potentiële kandidaat voor de behandeling van deze patiënten met een STAT-1 GOF-mutatie. Vóór de start met baricitinib leed de beschreven patiënt continue aan orale en slokdarm schimmelinfecties (candidiasis). Chronische behandeling met profylactische antischimmelmedicatie was noodzakelijk want anders ontwikkelde de patiënt in twee weken tijd wederom candidiasis. De patiënt ontwikkelde verder ook orale en vaginale ulceraties waarvan werd aangenomen dat ze ontstonden op basis van auto-immuniteit. De behandeling met baricitinib resulteerde in een sterke klinische verbetering. Profylactische antischimmelbehandeling en immunosuppressieve therapie konden worden gestopt zonder nieuwe verschijnselen van candidiasis of ulceraties. Baricitinib verlaagde ook de hyperfosforylering van STAT1 en verhoogde de productie van IL-17A, IL-17F en IL-22 door de witte bloedcellen van de patiënt. Gedurende de behandeling was de expressie van de door STAT1 gereguleerde genen CXCL9 en CXCL10 verminderd en benaderde het niveau van gezonde controles. Op basis van deze bevindingen hebben we voor de eerste keer de therapeutische effectiviteit van baricitinib aangetoond bij een patiënt met STAT1 GOF-mutatie.

We hebben verder ook het positieve effect aangetoond van baricitinib bij een patiënt met het Aicardi-Goutières-syndroom (AGS), dit zijn patiënten met een SAMHD1-mutatie, zoals beschreven in **hoofdstuk 4**. De beschreven patiënt had ernstige klinische symptomen zoals doorbloedingsstoornissen aan handen en voeten. De ontstekingen en pijn verergerden sterk na blootstelling aan koude. Binnen 6 weken na de start van baricitinib was het probleem van de "winterhanden/voeten" opgelost en dit persisteerde 2 jaar lang. De laesies kwamen dan ook niet terug in de winter. Vijf van de door interferon type I geactiveerde genen (ISG's;

IFI44, IFI44L, IFIT3, LY6E en MX1) die de "handtekening" vertegenwoordigen van (verhoogde) type I IFN activiteit, werden gemeten als marker voor ziekteactiviteit. De verhoogde ISG's niveaus zoals gemeten vóór de start van baricitinib, daalden aanzienlijk tijdens de behandeling. De hyperfosforylering van STAT1 welke werd gemeten in de T-lymfocyten vóór de behandeling met baricitinib was ook duidelijk afgenomen. Onze bevindingen suggereren dat baricitinib een veelbelovende nieuwe kandidaat is voor de behandeling van AGS-patiënten.

Nieuwe STAT3 GOF-mutaties worden beschreven in **hoofdstuk 3** van dit proefschrift. We beschrijven twee nieuwe STAT3 activerende mutaties (p.[Y360C]) en p.[L387R]) in twee families. Van elke familie waren twee patiënten onder behandeling. De klinische symptomen van de patiënten omvatten immuundeficiëntie, auto-immuunverschijnselen, groei en dysmorfe kenmerken en chronische longziekte. Alle patiënten hadden verhoogde, door STAT3-gereguleerde cytokines IL-6 en IL-17. In vitro studies met getransduceerd HEK293-cellen met de heterozygote STAT3-mutaties lieten een defect transport van het wild-type (WT) STAT3 naar de kern zien, wat suggereert dat de activerende mutaties een negatief effect hebben op transport van WT STAT3. De STAT3 mutaties veroorzaken een verhoogde transcriptie van STAT3 afhankelijke genen zoals aangetoond met luciferase reporter-assays, en ook toename van STAT3 "downstream" gereguleerde genen zoals SOCS3. Een tot nu toe niet bestudeerde functie van STAT3 in afweerstoornissen is het niet-klassieke effect van STAT3 op mitochondriën. Dit werd verricht op mononucleaire witte bloedcellen en toonde een verhoogde functie van de mitochondriën in het geval van de twee GOF-mutaties. Deze mutaties zijn ook geassocieerd met vermindering van naïeve T-lymfocytenpopulatie en vroege uitputting van T-lymfocyten. Verschuiving van de lymfocytenpopulaties zou ook een bijdrage kunnen leveren aan de veranderde mitochondriale functies omdat de verschillende subtypen lymfocyten een potentiele verandering in metabolisme ondergaan tijdens differentiatie.

Er werd een behandeling gestart met tofacitinib, ook een JAK-remmer, van de twee patiënten met de Y360C STAT3 GOF-mutatie die in een gevorderd stadium van ziekte verkeerden. Bij een van de patiënten met refractaire pulmonale hypertensie werd vrijwel geen effect van tofacitinib waargenomen. Bij de andere patiënt met Y360C STAT3 GOF-mutatie verbeterden haar klinische verschijnselen gedurende de behandeling. De pulmonale hypertensie bleef onder controle tijdens de behandeling met tofacitinib want deze recidiveerde niet. Bovendien verdween de

ADDENDUM

Acknowledgements

Curriculum Vitae

PhD Portfolio

cachexie tijdens de behandeling.

In **hoofdstuk 5.1-5.2** wordt de functie van het STAT-eiwit in termen van biologie en klinische consequenties bediscussieerd. De gelijkenis van alle STAT-moleculen, competitie voor dezelfde doelwitgenen en heterodimeervorming worden gepostuleerd als de mechanismen die verantwoordelijk zijn voor de overeenkomstige klinische fenotypes die worden gevonden bij zowel STAT-functieverlies (LOF) en bij GOF-mutaties.

In **hoofdstuk 5.3** worden de behandelingsmogelijkheden beschreven die zijn gericht op de interactie met de JAK/STAT-signaalroute, zoals ook in onze studies toegepast. We bespreken verder nieuwe ontwikkelingen die moeten uitmonden in remming van de JAK/STAT-signaalroutes, om zodoende te komen tot effectievere therapieën met een acceptabel bijwerkingenpatroon.

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my promotor, Prof.dr. P.M. van Hagen, my supervisors, Dr. V.A.S.H. Dalm and Dr. W.A. Dik., and Prof. dr. Nattiya Hirankarn. I would not have an opportunity to be a PhD student in the Netherlands without you. In 2014, when I was searching for a university with a PhD program, I met all of you (Martin, Wim, and Virgil) for the first time in Thailand. At that time, talking to foreigner was already worrisome for me. With helps from Nattiya, Martin (and two other unfortunate supervisors) accepted me for PhD program at Erasmus MC. I remembered that I kept checking my email every hour looking for the confirmation messages. It was such a great honor to work with all of you and being your student. I have to say that in these four years as a PhD student, I really have huge developments, in terms of scientific research, laboratory skill, and personal attitude.

I am deeply grateful to Prof.dr. Nattiya Hirankarn, Ajan Ple. You encouraged me to go for PhD and put effort on finding PhD program for me. In the first few months of my PhD, I called you from Rotterdam and told you that I cannot handle PhD life abroad anymore, my life in Rotterdam was too tough to survive for four years. You told me to be patient and it takes time to adapt to everything. And it's true. If I gave up on that day, I would not be sitting here writing acknowledgement in my thesis book. Thank you so much for your trust and your support. You are my model for being a nice supervisor and teacher.

I'm so thankful for my promotor, Martin. In science, you are very creative and full of fresh amazing ideas. Every meeting we had, you raised up new and ideas which gave us opportunities to think about new approaches in our research topics. As a supervisor, you have always given a hand whenever I have unmanageable problems. You can really deal with anything. And here, I also would like to thank Judy, Martin's right-hand lady who help me out with all the documents and fuzzy procedures.

Dear Virgil, although I met you averagely once a week, you were the supervisor who I felt close to the most. I have to admit that I pretty scared of you when I met you for the first time in Bangkok. You looked quite serious and tough, which I found later that you are not as serious as I imagined. You always told me to be positive about stuff which you probably think that it never worked. However, whenever I had meeting with you, I usually felt better with my work progression

and also with myself, for somehow. You made me see the good sides of whatever I was doing. You normally did not speak a lot in the meeting with Wim and/or Martin but you, actually, always had brilliant ideas. I truly appreciate your thoughts as well as your plans in every experiment, especially when we need to work with patients.

Dear Wim, since I mostly worked in the lab, you are the supervisor I contacted the most. On the very first days as a PhD student, I heard a lot about how strict and scary you are. I remembered that once I had a question and someone told me "You can ask Wim. Do you dare?" (and of course, I didn't ask you). However, after I started working with you, I never think that you are a strict person. Actually, I think you are really reasonable and sympathy. Among the three supervisors, you stayed with me almost every time that I cried (this doesn't mean that you made me cry) and you made me feel better. You also amaze me your ideas in experiments. Whenever I thought I had bad results and walked sadly into your offices, you could always find something hopeful in them. I am also truly grateful that you always put so much efforts and ideas on our works to aim for the best as they could be. Without you, we will never be able to have our works published in high impact journals.

Honestly, being a PhD student in the Netherlands was the most difficult four years of my life. Far away from my familiar places, people, and home in Bangkok, it was extremely challenging to settle down here. Stress and depression were all around me at the beginning of my PhD life. Therefore, I want to say thank you for all my Thai colleagues in Rotterdam (Hello Kariang!). Keng, I really appreciate for all the things you did for me. You helped me a lot and were around me no matter what happen. You are the biggest helps for me and make my life in Rotterdam much more enjoyable. We passed through all the tough times together. I am so sorry that you have to stay a bit longer than me in Rotterdam, but I'll still be around you as always. I believe in you that you will hit a home run very soon and I'm looking forward to working with you in Chulalongkorn again. P'Karn, funniest unfunny guy, you make me laugh like crazy every time we hung around together. It was so cool that we (also with Keng) can get along with each relatively much better than with anyone else. Anyway, thank you so much for you help and advice both in the lab and in life. Thank you for late night dinners at the China Town (also with Zhong Li). Thank you for being the joy of my Rotterdam life. Dear P'Utt, Syriam, Jan, Kib, and P'Ply, it's always nice to going out with you. I will miss BBQ in the Park with all of you. Every dinner and trip we had together cheered me up and energized me. It was a great pleasure to meet all of you in Rotterdam and I was in regret to leave

you. You still have your battle to fight, just be patient and keep moving. Every dog has his day.

As I came directly from a bachelor's in medical doctor degree, working in the laboratory was something new to me. Without the tremendous help from Benjamin, I would probably jump out of the lab window with in the first year. I cannot thank you enough for your assistance and efforts. Honestly, you were always the first one in the lab I would run to, when I had results (either good or bad) from my experiments. Your advices always mean something to me. We spent several nights (or I should say morning, haha) together behind flow machine or flow cabinet. No matter how late we had to stay for the experiments, you never gave up. We got a number of Kaiser points, although we do not really know how to claim these points and what are they for. I really appreciate everything you have done for me. You also my model for zero-waste lifestyle and to eat less meat (being vegetarian is still too difficult for me). It was funny that I decided to have a pack of mango for breakfast instead of a bacon-cheese croissant after you introduced me to watch Okja. You are a truly great man with an extremely good heart. I was so lucky that I met you and once being your colleague.

Dear Pamela, If I could say only one sentence to you, I would ask you that why did we just met in my last year of PhD? Working with you was a pleasure. You helped me a lot, both in the lab and outside. I truly appreciate your helps which made me pass through the challenging time of my PhD. Having you around me made everything I had to do more delightful. Working in the lab had never been so fun. I remember when we chitchatted about stuff til we went back home late. You also helped me preparing my room for the moving out and that really cheered me up much more than when I had to do it alone. You were the only one in the lab who ever asked me if I had something to eat for my dinner and offered to bring me food (and gumdrops >v<). I would like to thank you for being both my colleague and my friend. Although we were around together for only about half a year, it was really a good time. I'm glad that I met you. Maybe I am not your lazy boss (haha) anymore, but you are my friend, forever.

Conny! How can we work without you? You know the best where all the stuff in the lab are and always manage to arrange everything nice and tidy! Thank you so much for every help and advice. ELISA queen did give part of her power to me. Nicole, I truly appreciate your help especially with the flow cytometry. You taught me how to work with it and I did need to do flow in every of my project.

Maaike, I'm so thankful for your help in RQ-PCR and also other stuff. You did work hard for me, and everything turned out brilliant! IRD group colleagues, I'm glad that once I was part of this team. I'm so thankful for all of your help and kindness. I will miss working in the lab with all of you.

Fabian, Super Chang! You helped me with a lot of things in the lab. I really appreciate everything you had done for me. Your advice always made me learn something new. You are the most hard-working student I have ever met, and you are handy with almost every tool and process in the lab. I believe that one day you will be a great scientist as well as a great doctor. Your success is approaching, just keep going and be patient. Please do not forget that Chang never give up!

Christopher, your calm and kindness make you the most amazing person. Ever since I have known you, you made me smile or laugh every time we met, even within a few minutes at the hallway. You know plenty of techniques in the lab and you willing to help out wherever there is a need. I thought that if there are so many people bother me the way I and other people did to you, I probably feel annoyed. On the contrary, you never looked irritated at all. Your office door never closed. Your thoughtful and kindness are my model and I want to be a great scientist like you.

Dear my officemates Marieke, Iris, Jamie, and Steven, it was nice to meet you guys. Marieke, we shared the same office since I started working in EMC. I am sorry that I missed your promotion day. I am sure you are doing great with your new job. Iris, you started your PhD almost at the same time with me. I remembered you taught me to do PBMC isolation and I still used your protocol until now. At the beginning, I did not speak a lot to anybody. You were almost the only one in the lab I talked to. Since we get familiar with each other, we did chitchat a lot in the office :) Jamie and Steven, I hope you are doing well. Your diligence and your patient will bring you success. You were always nice to me and we helped each other whatever we can do in our office. Iris, Jamie, and Steven, I will not forget our great times at Takumi and Shabu Shabu. I hope when I have an opportunity to go back to Rotterdam again, we will have time to dine out together somewhere.

Chris and Jenna, you are among the persons I must say that I was so lucky to meet you. I had a hard time at the beginning of my PhD, and you did help me with all your advice and your friendliness. Jenna, you were nice to everyone and it was sad to see you leave EMC while Chris and I still had to work in the lab. I very much appreciate your help on flow cytometry and also other stuff. Chris, you were

always nice to me. It was fun that we shared matcha treats with each other. I hope you have a plan to visit Bangkok again. There are plenty of tea bars and dessert cafes pop up that I want to go with you.

Hanna Kok, although you left EMC not so long after we met, we had a very good time together. You taught me to do transfection and it did work very well. We went to cinema together which was my first time in Rotterdam. I hope you are doing great at your current work place. I am still stalking you on Facebook and Instagram ;)

Astrid, we met only half a year before I left EMC. You are such a cheerful person with a thoughtful mind. Talking to you did cheer me up. It was a pity that we did not go to the museum together but at least we spent good time at our workplace. I am glad that I could help you out with some tiny little things in the lab which also made me feel good to myself. I think I cannot go to your graduation day :(sorry, but I am sure that you did brilliant jobs in your study.

Olivia, we had a fantastic time in Lisbon! Thank you for hanging around with me there. Everything was so nice. You still need to work with Virgil, sorry for that, haha (just kidding). I'm sure you will do very well in your PhD. Looking forward to seeing you around again soon in the congress somewhere.

Jorn, the Lion! Having you around in the lab was so fun. Martijn, you are the politest Dutch guy I ever met (in contrast to Jorn, haha). Both of you are nice guy. Thank you so much for being around and helping me with all the stuff. You are very smart and I'm sure you will do great in your PhD.

Dear all my colleague in Department of Immunology ErasmusMC, I want to take this opportunity to thank all of you for your support and care you had given me while we worked together. It was a big adaptation for me to work there, at the beginning. I have to learn numerous of new things and I would never be able to do it without your (either direct or indirect) guidance and help. I tried to be nice to everyone as much as possible and I'm sorry if I did disappoint any of you for whatever reasons. Thank you for making this workplace such an amazing place that gave me plenty of experiences. I have already missed all of you. Working without anyone of you is never be the same. I promised that if I have an opportunity to go to the Netherlands or nearby, I will definitely visit you all again.

Dear my friends and colleagues in Thailand, although our life, times, and all

the situations have changed over the years, but our wonderful bonding stays. Thank you so much for your support and trust in me. All the good time we spent together were one of the drives for me to finish my PhD as soon as possible and come back to see you again in Thailand.

Here, I would like to also express my special gratitude to my collaborators, Peter J. van der Spek, Sigrid MA Swagemakers, Chiara Milanese, Pier G. Mastroberardino, Angelique van Rijswijk, Sandra J. Posthumus-van Sluijs, Cornelia G. van Helden-Meeuwsen, Marjan A. Versnel, Emilia K. Bijlsma, Claudia A. L. Ruivenkamp, Margreet J. Oele, Deon J. Venter, Narissara Suratannon, and other collaborators for your valuable support and contribution in this thesis.

Last but not least, I would like to thank my family who always support me. We not usually say love to each other, but I want you to know here that you are the most crucial persons in my life. Mom and Papa, you worked hard together since I was very young to provide me excellent educations. Although I had to study until late several days per week and also on the weekends, you were always by my side. No matter what I want to study and try, you always support me, and we aim for the best. This make me learn to commit myself to excellence. Parenting a child to grow up to be a strong and successful adult is extremely difficult, but I think you did it very well. Without you, it is impossible for me to be here. You are truly the wind beneath my wings.

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Education

2006-2012:	Faculty of Medicine, Chulalongkorn University Doctor of Medicine (GPAX 3.24)
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2000-2003:	Patumwan Demonstration School, Srinakharinwirot University (GPAX 3.92)
1994-2000:	Rajini School (GPAX 4.00)

Certifications and Licenses

2014: Certificate of attendance in "Standard Course in Clinical Trials", Faculty of Medicine, Chulalongkorn University.

2012: Certificate of achievement in one-year internship program after M.D., Ministry of Health, Thailand.

2012: Doctor of Medicine, Chulalongkorn University.

2012: Medical license, the Medical Council of Thailand.

2004: Certificate of Merit Class: High Distinction in the Senior Division of the 2004 Australian National Chemistry Quiz.

2004: Pass the selection and complete the 1st level of training program in biology from The Promotion of Academic Olympiad and Development of Science Education Foundation under the patronage of Her Royal Highness Princess Galyani Vadhana Krom Luang Naradhiwas Rajanagarindra.

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Work/Educational Experiences

2015 – current: PhD Student of Erasmus University Medical Center Rotterdam, the Netherlands

2013 – 2015: Internship/teaching assistant in Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

2013 – 2014: Guest lecturer: Preparation for Thai Medical Licensing Examination (Step 1) for medical students in Surin Hospital.

2013: Co-organizer in 1st Medical Mycology Training Network, Thailand.

2013: Co-doctor in research project "Effectiveness of fermented milk containing probiotics in reducing risk of acute respiratory tract infection in children"

2012: General physician at Hua Hin Hospital, Prachuap Khiri Khan and Kratumban Hospital, Samut Sakhon.

2010: Part of the medical student working group for improving the health care accessibility of elderly people around Phanatnikhom Hospital, Chonburi.

2010: Part of the medical student group for research analysis "Outcomes after an excisional procedure for cervical intraepithelial neoplasia in HIV-infected women" retrospective cohort study.

Publications

- A Novel Heterozygous Mutation in the STAT1 SH2 Domain Causes Chronic Mucocutaneous Candidiasis, Atypically Diverse Infections, Autoimmunity, and Impaired Cytokine Regulation. Meesilpavikkai K, Dik WA, Schrijver B, Nagtzaam NM, van Rijswijk A, Driessen GJ, van der Spek PJ, van Hagen PM, Dalm VA. Front Immunol. 2017 Mar 13
- Diversity and Antifungal Drug Susceptibility of Cryptococcus Isolates in Thailand.Worasilchai N, Tangwattanachuleeporn M, Meesilpavikkai K, Folba C, Kangogo M, Groß U, Weig M, Bader O, Chindamporn A. Med Mycol. 2016 Dec 3
- Baricitinib treatment in a patient with a gain-of-function mutation in signal transducer and activator of transcription 1 (STAT1). Meesilpavikkai K, Dik WA, Schrijver B, Nagtzaam NMA, Posthumus-van Sluijs SJ, van Hagen PM, Dalm VASH. J Allergy Clin Immunol. 2018 May 2.
- Efficacy of baricitinib in the treatment of chilblains associated with the type I interferonopathy Aicardi-Goutières syndrome. Meesilpavikkai K, Dik WA, Schrijver B, van Helden-Meeuwsen CG, Bijlsma EK, Ruivenkamp CAL, Oele MJ, Versnel MA, van Hagen PM, Dalm VASH. Arthritis Rheumatol. 2019 Jan 22.

Extracurricular/Volunteer Experiences

2010: Secretary of The Student Union of the Faculty of Medicine, Chulalongkorn University (SMCU).

2010: Responsible in the project "Wai Kru Ceremony" (A ceremony to pay respect to the teachers).

2010: Participate as a volunteer in the health care and preventive medicine for people in border and hardship areas in Nakhon Si Thammarat province, organised by the Princess Mother's Medical Volunteer Foundation.

2010: Participate as a volunteer in the project to prevent H1N1 influenza for the public, organized by SMCU at Siam Square, Bangkok.

2010: Participate as a medical first aid staff of the projects organized by

the Student Government of Chulalongkorn University.

2007-2010: Co-organizer of the pre-admission camp for high school students interested in studying medicine, organized by SMCU.

2008: Quiz committee of the Medical Biology Competition for high school students in Chula-Vichakarn (Chulalongkorn University Academic Expo).

2007-2008: Participate as a volunteer in tuition camp for high school students at Sakon Nakhon, Bangkok and Kalasin provinces (3 camps)

2007-2008: Co-organizer of the SMCU project to welcome the freshmen.

2006-2008: Co-organizer of the CU Singing Contest project, organized by SMCU.

2006: Participate as a volunteer in the project to distribute the medical knowledge in Thai Red Cross Fair, Bangkok.

2006: Participate as a volunteer in the project to distribute the medical knowledge in the safe sex project to celebrate the 90th Anniversary of Chulalongkorn University.

2006: Participate in swimming and water polo competition in the 1st year student sport game, winning 1 bronze medal.

2001: Intermediate Piano Certificate (5th level) by the International Examination Board; Pass, Trinity College London.

PhD PORTFOLIO

Summary of PhD training and teaching

Kornvalee Meesilpavikkai

Erasmus MC, Department of Internal Medicine, Department of Immunology

Research School: MolMed Erasmus University

PhD period: 2015-2019

ACTIVITIES	YEAR	WORKLOAD (ECTS)
Courses		
Introduction in GraphPad Prism	2015	0.3
Annual Course on Molecular Medicine	2016	0.7
CC02a course Biostatistical Methods I: Basic Principles Part A (Nihes)	2016	2
XVth Course Biomedical Research Techniques	2017	1.5
Scientific integrity course	2017	0.3
Biomedical English Writing & Communication	2017	3
Ensembl Gene Browsing workshop	2018	0.6
Advance Immunology Course	2018	4.5

ACTIVITIES	YEAR	WORKLOAD (ECTS)
Presentations and (inter)national conferences		
Poster presentation in the International Primary Immunodeficiencies Congress (IPIC), Budapest, Hungary	2015	1
Presentation in international research meeting between Faculty of Medicine Chulalongkorn University and Erasmus University Medical Center International Symposium 2015: Nephrology, Dermatology, Endocrinology and Immunology, Bangkok, Thailand	2015	1
Poster presentation in Wetenschapsdagen 2016, Antwerp, Belgium	2016	1
Poster presentation in 20th Molecular Medicine Day 2016, Rotterdam, the Netherlands	2016	1
Poster presentation in 17th biennial meeting of the European Society for Immunodeficiencies (ESID), Barcelona, Spain	2016	1
Oral presentation in 10th European Workshop on Immune-Mediated Inflammatory Diseases (EWIMID), Toulouse, France	2016	1
Poster presentation in Wetenschapsdagen 2017, Antwerp, Belgium	2017	1
Poster presentation in 21th Molecular Medicine Day 2017, Rotterdam, the Netherlands	2017	1
Presentation in Dutch WID Immunology Expert Meeting on PID 2017, Santpoort, the Netherlands	2017	1
Presentation in Internal Medicine Research Symposium	2017	0.4
Poster presentation in the International Primary Immunodeficiencies Congress (IPIC), Dubai, Emirates	2017	1

ACTIVITIES	YEAR	WORKLOAD (ECTS)
Attend the Second International Primary Immunodeficiency Meeting: From Bench to Bedside, Chulalongkorn University, Bangkok Thailand	2017	1
Poster presentation in Wetenschapsdagen 2018, Antwerp, Belgium	2018	1
Poster presentation in 22th Molecular Medicine Day 2018, Rotterdam, the Netherlands	2018	1
Poster presentation in 20th Congress of the International Society for Human and Animal Mycology, Amsterdam, the Netherlands	2018	1
Attend Diagnostics in Medical Mycology Workshop, Faculty of Medicine Chulalongkorn University, Bangkok, Thailand	2018	1
Poster presentation in 18th biennial meeting of the European Society for Immunodeficiencies (ESID), Lisbon, Portugal	2018	1
Poster presentation in the 7th Federation of Immunological Societies of Asia-Oceania Congress (FIMSA 2018), Bangkok, Thailand	2018	1
Teaching		
Histology teaching (32 hours)	2015	1.1
Histology teaching (34 hours)	2016	1.2
Histology teaching (36 hours)	2017	1.3
Infection and Immunity student rotations (8 hours)	2017	0.3
Histology teaching (20 hours)	2018	0.7
Others		
Journal club	2015-2018	1
Department seminars	2015-2019	1
Total ECTS		**36.9**