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Immune dysregulation as a consequence of genetic variants within the JAK-STAT signalling pathway

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Abstract

Research in immunology has been a rapidly evolving field in recent years, largely facilitated by the advent of next-generation sequencing techniques. The opportunity to study the entire coding sequence of the genome has resulted in the discovery of more than 400 well-characterized, monogenic inborn errors of immunity. Research on these rare patients, who mostly present early in life to pediatric hospitals, has informed our understanding of human immunity since it allows to study the function of a mutated gene product in the context of human disease.

Whilst the power of the immune system has evolved to fight infection, fine-tuned regulatory mechanisms gained importance. Keeping the balance between tolerance and protection involves close communication between immune cells and their environment. Such interactions are often mediated by soluble factors, i.e. cytokines.

The work presented in this thesis is centered around two important signalling pathways: Interleukin-2 signalling has long been recognized pivotal for T cell immunity. The discovery of homozygous loss-of-function variants in *IL2RB* underscores its non-redundant role in preventing autoimmunity by promoting regulatory T cell survival and function. Additionally, a terminal differentiation defect of cytotoxic lymphocytes renders affected patients specifically susceptible to *Cytomegalovirus* disease. Additional work on patients with loss- and gain-of-functions in the downstream signalling molecule STAT5B expands the phenotypic spectrum and explores potential treatment options using Janus tyrosine kinase inhibitors.

The second pathway under study is type I interferon signalling. Functional validation of a new biallelic *IFNAR1* variant abrogating responses to type I interferon was undertaken in an individual presenting with haemophagocytic lymphohistiocytosis following receipt of live-viral vaccine. Lethal autoinflammation was also seen in patients where homozygous STAT2 variants were found to hamper negative feedback regulation and thus causing unrestrained type I interferon activity. Mechanistic studies on the inflammatory consequences of dysregulated type I interferon responses in STAT2- and IRF9-deficient primary cells and induced pluripotent stem cell derived macrophages offer a molecular explanation for the clinically observed inflammation in these individuals.

By dissecting the molecular mechanisms underlying immune dysregulation in these rare patients with monogenic errors of immunity I am hoping to contribute to improved diagnostic rates and to help pave the way for personalized treatment options in these ‘orphan’ diseases.

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7-AAD:	7-Aminoactinomycin D
AD:	Autosomal dominant
ANOVA:	Analysis of variance
BMP-4:	Bone morphogenetic protein 4
Bp:	Base pair
BSA:	Bovine serum albumine
CADD:	Combined annotation dependent depletion
CCD:	Coiled-coil domain
CD:	Cluster of differentiation
CMV:	Cytomegalovirus
CNS:	Central nervous system
CRISPR:	Clustered regularly interspaced short palindromic repeats
CSF:	Cerebrospinal fluid
CTLA4:	Cytotoxic-T-lymphocyte associated protein 4
DAPI:	4',6-Diamidino-2-phenylindole
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EBV:	Epstein Barr virus
EDTA:	Ethylenediaminetetraacetic acid
EMCV:	Encephalomyokarditis virus
FACS:	Fluorescence-activated cell sorting
FcεRIγ	High affinity immunoglobulin ε receptor subunit γ
FCS:	Fetal calf serum
FMO:	Fluorescence minus one
FOXP3:	Forkhead box P3
GAF:	γ-activating factor
GAS:	Interferon γ activation sites
GFP:	Green-fluorescent protein
GOF:	Gain-of-function
HLA-DR:	Human Leukocyte Antigen-DR isotype
HLH:	Haemophagocytic lymphohistiocytosis
HRP:	Horseradish peroxidase
HRS:	Hours
HSCT:	Haematopoietic stem cell transplantation
HSV-1:	Herpes simplex virus-1
ICAM1:	Intercellular adhesion molecule 1
IEI:	Inborn error of immunity
IFN:	Interferon

IFNAR:	Interferon α receptor
IFNGR:	Interferon γ receptor
IFNLR:	Interferon λ receptor
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IL2Rα:	α-subunit of the IL-2 receptor
IL2Rβ:	β-subunit of the IL-2 receptor
IL2Rγ:	Common γ-chain
IPEX:	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iPSC:	Induced pluripotent stem cell
IRF1:	Interferon regulatory factor 1
ISG:	Interferon stimulated gene
ISGF3:	Interferon stimulated gene factor 3
ISRE:	Interferon-sensitive response element
JAK:	Janus tyrosine kinase
JAKinib:	Janus tyrosine kinase inhibitor
KLRC2:	Killer cell lectin like receptor C2
KO:	Knock-out
LCL:	Lymphoblastoid cell line
LOF:	Loss-of-function
LRBA:	Lipopolysaccharide-responsive and beige-like anchor protein
M-CSF:	Macrophage colony stimulating factor
MMR:	Measles, mumps, rubella
MRI:	Magnetic resonance imaging
mRNA:	Messenger RNA
NA:	Not applicable
NKG2C:	Killer cell lectin like receptor C2
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline
PCA:	Principal component analysis
PCR:	Polymerase-chain reaction
PD-1:	Programmed cell death protein 1
PE:	Phycoerythrin
PI3K:	Phosphoinositide 3-kinase
PIAS:	Protein inhibitor of activated signal transducer and activator of transcription
PICU:	Paediatric intensive care unit
PLZF:	Zinc finger and BTB domain-containing protein 16
PMA:	Phorbol myristate acetate
RIG-I:	Retinoic acid-inducible gene I
ROCK:	p160-Rho-associated coiled-coil containing protein kinase
RoV:	Rotavirus

RPMI: Roswell Park Memorial Institute 1640 medium
RNA: Ribonucleic acid
RT-qPCR: Reverse transcription-quantitative PCR
SARS-CoV2: Severe acute respiratory syndrome coronavirus 2
SCF: Stem cell factor
SCID: Severe combined immunodeficiency
SDS: sodium dodecyl sulfate
SHP: Src homology region 2 domain-containing phosphatase
SOCS: Suppressor of cytokine signalling
STAT: Signal transducer and activator of transcription
STING: Stimulator of interferon genes
SYK: Spleen tyrosine kinase
TBST: Tris-buffered saline containing tween
T_{CM}: Central memory T cell
T_{eff}: Effector T cell
T_{EM}: Effector memory T cell
T_{EMRA}: Effector memory T cell re-expressing CD45RA
TF: Transcription factor
TGF-β: Transforming growth factor β
T_{H2}: T helper cell type 2
TIGIT: T-cell immunoreceptor with Ig and ITIM domains
TLR: Toll-like receptor
T_N: Naïve T cells
TNFα: Tumour necrosis factor α
T_{reg}: Regulatory T cell
T_{SCM}: Stem cell memory T cell
T_{TE}: Terminal effector T cell
TYK2: Tyrosine kinase 2
USP18: Ubiquitin-specific protease 18
VEGF: Vascular endothelial growth factor
WES: Whole exome sequencing
XLA: X-linked agammaglobulinaemia

1. Introduction

1.1 Inborn errors of immunity

Throughout most of human history, life-expectancy at birth was limited to 20-25 years with infectious disease being the major cause of childhood death[1]. When Robert Koch identified *Mycobacterium tuberculosis* in 1882, a causative link between pathogen and disease could be established for the first time[2]. At this point, infection was thought to be synonymous with disease and the third of Koch's postulates states that the isolated and cultured microorganism should cause the same disease when administered to a heathy organism[3]. But only a few years later it became apparent that a large proportion of people infected with *M. tuberculosis* or *Streptococcus pneumoniae* remain asymptomatic[4]. Such inapparent infections could be explained by a concept where previous infections may have resulted in specific immunity enabling the host to keep the pathogen dormant. However, this theory could not sufficiently account for interindividual variability seen in the course of primary infection, for example, the fact that fevers killed mostly children, with far fewer deaths seen in the elderly[4]. In the 1930s it was proposed that the germline genetic background of the host might influence susceptibility or resistance to pathogens and the term "inborn error of immunity (IEI)" was introduced[5].

The first description of such a suspected primary immunodeficiency was published in 1950 by Rolf Kostmann[6]. He reported 14 children from Sweden, who almost all died within their first year of life due to a variety of infections, mostly septic skin infections. Since the disease followed a recessive mode of inheritance and the peripheral blood of patients was found devoid of granulocytes, he termed the disease 'infantile genetic agranulocytosis.' Loss-of-function mutations in the *HAX1* gene were identified decades later as the underlying genetic cause of Kostmann's disease[7]. During the following years the hypothesis that life-threatening childhood infections are caused by single-gene IEI gained momentum: Autosomal recessive mutations in *UNC93B1* for example were found to underlie *Herpes simplex virus 1* (*HSV-1*) encephalitis in children[8]. In comparison to XLA, where affected patients are susceptible to a variety of pathogens for which humoral immunity is essential, *UNC93B1* mutations only cause a narrow hole in the immune defence of affected children rendering them specifically susceptible to *HSV-1* but leaving protective immunity against other pathogens intact.

In recent years and largely facilitated by the development of next generation sequencing technologies, particularly whole exome sequencing, over 400 monogenic IEIs have been described[9]. With expanding knowledge, it became clear that susceptibility to infection may not be the only feature of IEIs. Heterozygous gain-of-function (GOF) mutations in *signal transducer and activator of transcription 1 (STAT1)* gene for example were identified as the most frequent genetic cause of specific susceptibility to mucocutaneous infections with *Candida albicans*[10]. However, studying a large cohort of affected individuals, autoimmune features such as thyroid disease were noted in more than a third of patients[11]. To date, IEIs therefore not only include monogenic defects leading to immunodeficiency with increased susceptibility to certain pathogens, but also diseases where dysregulated immune responses result in profound autoinflammation, autoimmunity, or predisposition to malignancy.

1.2 JAK-STAT signalling

The Janus kinase (JAK)-STAT pathway is a well-studied signalling node connecting extracellular ligand-receptor interactions with transcriptional activity in the nucleus. More than 50 different cytokines, growth factors and hormones utilize this system emphasizing its central role in cell communication which is not limited to the immune system[12].

The association of a given cytokine with its corresponding transmembrane receptor leads to oligomerization of the cytoplasmic receptor tails where specific JAKs are non-covalently bound. There are four structurally related members of the JAK family: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2)[13]. Receptor engagement activates those kinases and provokes their reciprocal trans-phosphorylation thereby releasing their intrinsic catalytic activity. Subsequently, the JAKs phosphorylate the cytoplasmic domains of their associated cytokine receptors forming docking sites for specific STAT molecules. In a third phosphorylation step the JAKs then activate the respective STAT molecules[12]. Type I interferon (IFN) signalling may be used to illustrate this type of signalling cascade: Upon type I IFN binding, the two receptor subunits IFNα receptor (IFNAR) 1 and IFNAR2 form a ternary complex with the ligand. JAK1, connected to IFNAR2 and TYK2, linked to IFNAR1, use the close proximity to phosphorylate each other before primarily activating STAT1 and STAT2 molecules.

Altogether, there are seven members of the mammalian STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6). Originating from ancestral gene duplications, they share a characteristic protein structure and varying sequence overlap[14]. Exclusively located in the cytoplasm until activation, tyrosine-phosphorylation by the JAKs enables them to dimerize and subsequently enter the nucleus. There, the STAT molecules act as transcription factors directly engaging with thousands of DNA binding sites thereby controlling distinct transcriptional programs[12]. However, it is important to recognize some of the inherent complexities within this fairly straight-forward signalling cascade: Most cytokines activate several different STAT family members to varying degrees. In addition to homodimers, heterodimers and tetramers of specific STAT molecules can be formed[15], [16]. Depending on the activating cytokine, an individual STAT molecule can induce different sets of genes as exemplified by the role of STAT3 in myeloid cells: both Interleukin (IL)-6 and IL-10 use STAT3 as their principal signalling moiety, however, their downstream cellular effects are clearly distinct as evidenced by their respective pro- and anti-inflammatory activities[12]. Additionally, the negative feedback regulation initiated by the activation of a specific STAT molecule usually also exerts effects on other STAT family members highlighting the delicate balance of different STAT molecules within individual cells[17].

Mutations in both JAK and STAT molecules are known to cause various disease states in humans and can be classified as either germline or somatic. Another way of grouping them is according to their net effect on signalling resulting in either GOF or loss-of-function (LOF)[12]. In JAK1 for example, a germline compound-heterozygous LOF-mutation was reported in a patient susceptible to mycobacterial and viral disease[18] whereas a germline heterozygous GOF variant caused immune dysregulation and hypereosinophilia in a different kindred[19]. Somatic *JAK1* GOF variants, on the other hand, have been observed in acute lymphoblastic[20] as well as myeloid leukaemia[21]. Accordingly, germline LOF mutations in *STAT1* (either homozygous or heterozygous) are also associated with mycobacterial and viral disease since STAT1 acts downstream of type I and II interferon receptors both of which cooperate with JAK1[22]. In turn, immune dysregulation can also be a feature of germline *STAT1* GOF variants as already mentioned above.

Over the past decade, pharmacological JAK inhibitors ("Jakinibs") have been approved as a new class of drugs effective in various haematologic and autoimmune diseases[23]. Tofacitinib, one of the earliest Jakinibs licensed for clinical use in patients suffering from rheumatoid arthritis, competitively inhibits the ATP-binding site of JAK3 leading to reduced kinase activity (also halting JAK1 and JAK2 to a lesser extent)[23]. Ruxolitinib, another first-generation Jakinib primarily inhibiting JAK1 and JAK2, was initially approved to treat primary myelofibrosis but has also been successfully used in patients with activating variants in *STAT1* as well as *STAT3* [24]. Such personalized treatment decisions based on individual patients' genetic and functional conditions are prominent examples of modern precision medicine benefiting patients with rare IEIs.

1.3 Interleukin-2 signalling

1.3.1 Interleukin-2 function in lymphocytes

Interleukin-2 (IL-2) has been extensively studied over the last decades as it was the first cytokine to be molecularly cloned[25]. Originally termed "T cell growth factor", IL-2 had been observed to be critical for T cell proliferation in vitro as well as the induction of T cell memory and effector responses. IL-2 is produced by conventional T cells after engagement of the T cell receptor as well as the co-stimulatory molecule CD28[26], [27]. The corresponding IL-2 receptor consists of different subunits. Whereas the IL-2 receptor α (IL-2Rα, also called CD25) subunit determines receptor affinity, the IL-2Rβ (CD122) as well as the common γ-chain (CD132) contain intracellular domains essential to induce signal transmission to the nucleus[28]. It is important to note that every receptor subunit has its private, non-overlapping IL-2 binding site and that different combinations of subunits are capable of signalling[29]. All three subunits together constitute a high affinity receptor, which is present on activated T cells. In the absence of the α-subunit, IL-2Rβ and the common γ-chain signal with intermediate affinity and this receptor configuration is present on resting T cells and NK cells. Interestingly, transcription of IL2RA encoding the IL-2Rα-chain is not only induced by TCR activation, but also by IL-2 binding itself thereby constituting a positive feedback loop further amplifying T cell activation[30]. The extracellular domain of IL2Rα can also be cleaved and can be measured in serum as soluble CD25. This gives rise to a diagnostic test which is used to detect excessive T cell activation in hyperinflammatory states like haemophagocytic lymphohistiocytosis (HLH)[31]. Whereas IL-2Rα is specific to the IL-2 receptor, IL-2Rβ and the common γ-chain are not: IL-2Rβ is part of the IL-15 receptor whereas the common γ-chain participates in the IL-4, IL-7, IL-9, IL-15 and IL-21 receptors as well as the IL-2 receptor [32]. Following receptor engagement, JAK1 and JAK3 are phosphorylated and induce signal transduction mainly via STAT5A/B homo- and heterodimerization[33].

Since IL-2 was initially discovered to promote T cell activation and expansion, clinical trials evaluated its potential use in boosting antitumor responses thus making recombinant IL-2 the first cancer immunotherapy[34]. However, limited success in patients suffering from renal cancer and metastatic melanoma came at the cost of considerable toxicity including cytokine storms due to the high IL-2 dose administered[34], [35]. Nevertheless, targeting the IL-2 receptor in order to modulate immune responses has resulted in the development of different monoclonal antibodies: Basiliximab, a chimeric monoclonal antibody targeting CD25, is in clinical use to prevent rejection in solid organ transplantation[36]. Daclizumab, a humanized monoclonal anti-CD25 antibody, had been licensed to treat patients with relapsing remitting multiple sclerosis. However, due to severe inflammatory central nervous system complications, approval was withdrawn in 2018[37].

As early as 1993, when the first mouse model with a knock-out (KO) in the IL-2 gene was generated, researchers were surprised by the lack of infection susceptibility in these animals given the apparently crucial role of IL-2 in T cell immunity[38]. While about half of the animals died within in first weeks of life due to lymphoproliferation and severe anaemia, the surviving mice developed inflammatory bowel disease[38]. Two years later, descriptions of KO mouse models deficient for either the IL2Rα[39] or the IL2Rβ chain[40] were published and again multisystemic autoimmunity, but not classical immunodeficiency, was associated with high mortality. Hence it became clear, that IL-2 must have additional, non-redundant roles in controlling immune responses and maintaining self-tolerance[35].

1.3.2 Regulatory T cells

The special capabilities of a certain subset of cluster of differentiation (CD) 4^+ T-cells was first appreciated in mice: In 1995, Sakaguchi *et al.* identified $CD25^{high}$ $CD4^+$ T-cells as being able to suppress autoimmunity in thymectomized mice[41]. Whilst not themselves proliferating in response to T-cell receptor stimulation *in vitro*, these cells suppressed expansion of naïve $CD4^+$ T-cells[42]. Since the α-subunit of the IL-2 receptor is generally upregulated in response to T-cell stimulation as outlined above, it was not clear whether $CD25^{high}$ cells indeed represent a distinct T-cell lineage or rather a particular state of activation[42]. The transcription factor forkhead box P3 (Foxp3) was subsequently found to be stably expressed at high level within these regulatory T-cells (T_{reg}s). Acting as a master transcriptional regulator, murine Foxp3 is essential for both the development of T_{reg}s in the thymus and their suppressive function in the periphery[43], [44].

T_{reg}s exert their suppressive function in different ways: They express various co-inhibitory surface receptors such as cytotoxic-T-lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) or T-cell immunoreceptor with Ig and ITIM domains (TIGIT), which interfere with TCR signalling and co-stimulation[45]. T_{reg}s also modulate immune responses by secreting anti-inflammatory cytokines such as IL-10 or transforming growth factor β (TGF-β)[46]. Given the constitutive expression of CD25, T_{reg}s are also able to assemble a heterotrimeric IL-2 receptor, which has high affinity and is therefore capable of limiting the availability of IL-2 for activated conventional T-cells rendering them susceptible to activation-induced cell death[47]. Finally, they are able to limit activation by hydrolysing adenosine-triphosphate through their ectonucleotidase CD39 [48].

1.3.3 T_{reg}opathies

The essential role of T_{reg}s in maintaining self-tolerance and regulating immune responses was reinforced when patients harbouring LOF mutations in the *FOXP3* gene were identified[49]. These individuals lack functional T_{reg}s and usually present with multifaceted autoimmunity during the first year of life[50]. The acronym IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) reflects the most common manifestations: enteropathy with watery diarrhoea, eczema and autoimmune endocrinopathies such as insulin-dependent diabetes mellitus or hypothyroidism[50].

Three years earlier, a single individual with a homozygous 4 base-pair (bp) deletion in the *IL2RA* gene resulting in a complete lack of IL2Rα expression had been reported[51]. This boy, born to consanguineous parents, presented with lymphadenopathy, hepatosplenomegaly as well as chronic diarrhoea during the first year of life. In addition, infectious complications including Cytomegalovirus (*CMV)* pneumonitis and *Candida albicans* oeosophagitis were noted. Over the years,

very few additional patients with CD25 deficiency and similar clinical phenotypes have been identified[52]–[54]. These rare patients emphasize the critical importance of the high-affinity IL-2 receptor to the suppressive capacity of T_{reg}s.

In clinical immunology practice, patients presenting with severe autoimmunity but expressing wild-type *FOXP3* alleles have been classified as “IPEX-like”. Within this cohort, several other monogenic IEIs were discovered subsequently, and the shared feature of T_{reg}-dysfunction gave rise to the name “T_{reg}opathies”[45]. Heterozygous LOF variants in CTLA-4 cause immune dysregulation with hypogammaglobulinemia, lymphoproliferation and autoimmune cytopenias as frequent manifestations[55]. CTLA-4 normally enables T_{reg}s to inhibit T-cell co-stimulation by removing CD80 and CD86 from the surface of antigen-presenting cells[56]. Hence, these molecules are no longer available to interact with CD28 on the T-cell surface and such incomplete activation was shown to promote peripheral tolerance[57]. A clinically very similar IPEX-like syndrome is linked to autosomal-recessive mutations in lipopolysaccharide-responsive and beige-like anchor protein (LRBA)[58]. LRBA mediates recycling of CTLA-4 to the T-cell surface and prevents its lysosomal degradation. A loss of LRBA function therefore results in a secondary CTLA-4 deficiency[59]. Impaired regulation of CTLA-4 surface trafficking has also been observed in patients harbouring recessive variants in *DEF6* [60].

The *BACH2* gene encodes a basic region-leucine zipper transcription factor regulating high FOXP3 expression in T_{reg}s and therefore stabilizing their lineage identity and survival[61]. Reduced T_{reg} frequencies have been observed in human BACH2 haploinsufficiency presenting with lymphadenopathy, inflammatory bowel disease, and recurrent sinopulmonary infection[62].

Activating variants in STAT3 are also known to cause multi-organ autoimmunity early in life often manifesting as enteropathy, cytopenia, lymphoproliferation and interstitial lung disease[63], [64]. T_{reg} numbers may be normal or reduced in STAT3 GOF subjects, however, the molecular mechanisms underlying T_{reg} dysfunction are not fully understood as of yet. IL-6 driven STAT3 activation has been shown to inhibit the generation of T_{reg}s while promoting the differentiation of naïve T cells into IL-17 producing cells associated with autoimmunity in mice[65]. Additionally, STAT3 activation induces negative regulation via the transcription of suppressor of cytokine signalling (SOCS) 3. SOCS3 in turn also restricts STAT5 signalling known to positively regulate FOXP3 and CD25 expression[64]. In line with this, autosomal recessive LOF variants in STAT5B are also associated with reduced T_{reg} frequencies and severe immune dysregulation[66], [67]. Due to the characteristic short stature caused by interrupted growth hormone signalling which also depends on STAT5B, this IEI is currently not listed as a classical T_{reg}opathy but a “Combined immune defect with associated syndromic features” [68].

A recent cohort analysis studied 85 subjects suffering from IPEX-like symptoms without variants in *FOXP3*. In only 25% of these patients (21 out of 85), pathogenic variants in genes already linked to immune dysregulation could be identified, leaving three quarters of them without a molecular diagnosis[50].

1.3.4 Different roles of STAT5 molecules

Besides its role in transmitting signals from the IL-2 receptor to the nucleus, STAT5 serves as an intracellular messenger to various additional cell surface receptors. These include receptors for growth hormone, prolactin, and erythropoietin, as well as granulocyte-macrophage colony-stimulating factor [69]. Furthermore, all common γ-chain cytokines, and also IL-3 and IL-5, use STAT5

to induce transcriptional responses. Like the other STAT proteins, STAT5 enters the nucleus following its phosphorylation by JAKs and subsequent oligomerization. Besides homo- and heterodimerization, the research group of Warren J. Leonard has elucidated specific functions for STAT5 tetramers in both murine T and NK cells[70], [71]. Interestingly, STAT5A and STAT5B share 91% sequence identity on the protein level in both humans and mice[72] and their potentially non-redundant roles have been extensively studied using KO mouse models. *Stat5a*$^{-/-}$ mice show a profound defect of lactogenesis due to impaired prolactin signalling[73] whereas Stat5b-deficient mice experience insufficient growth hormone activity[74]. With respect to the immune system, the proliferative defect of thymocytes or peripheral T cells following IL-2 stimulation is more marked in Stat5b$^{-/-}$ mice and completely abrogated in double-KO mice[75], [76]. Furthermore, both Stat5 molecules contribute to murine NK cell development with again Stat5b-deficiency having a greater impact[75]. In summary, substantial data on STAT5 actions in the context of IL-2 signalling has been generated over the past decades. Nevertheless, important questions remain open, for example how diverse receptors induce different transcriptional responses via the same signal transducer (STAT5) or how the different STAT molecules regulate each other.

1.4 Interferon signalling

1.4.1 Types and function of interferons

The name interferon relates back to the concept of ‘viral interference’. During the first half of the 20th century, it was noted by different researchers, that the interaction of cells with either live or inactivated virus can induce a functional state, where the cells do no longer support the growth of a second virus added later on[77]. The soluble, virus-interfering substance was therefore termed interferon (IFN) [78]. Exploiting the antiviral properties of interferon for clinical applications soon became an important area of research[79]. However, initial studies in humans using impure type I monkey interferon did not meet the researchers’ hopes and expectations [80], [81]. In subsequent years, the molecular basis of IFN-activity was elucidated and tested in various model organisms. Three distinct families of IFNs became apparent: On chromosome 9, there are 13 genes encoding partially homologous subtypes of IFNα and additional genes encoding IFNβ, IFNε, IFNκ, and IFNω constituting the group of type I IFNs. The type II interferon family, conversely, only has a single member named IFNγ. In 2003, a third family consisting of 4 IFNλ-subtypes, encoded on chromosome 19, was described[82], [83]. With antiviral activity being the shared feature of all IFNs, there are considerable differences with regard to cell specificity and tissue activity. Type I IFN are produced by almost all nucleated cells following stimulation of pattern-recognition receptors by microbial products[84]. In contrast, the production of type II IFN is confined to certain lymphocyte subsets due to its potent immunostimulatory and possibly harmful potential. Type III IFN can again be produced by most cells but acts mostly on epithelial surfaces due to restricted receptor expression as detailed below[85].

1.4.2 Signalling cascade and regulation

Not only are all nucleated cells capable of producing type I interferon, the corresponding type I interferon receptor, consisting of the two subunits IFNAR1 and IFNAR2, is also ubiquitously expressed. Upon binding of any of the type I IFNs, the receptor subunits form a ternary complex with the respective ligand. JAK1, bound to IFNAR2 and TYK2, complexed to IFNAR1, use their close proximity to phosphorylate each other before activating STAT1 and STAT2 molecules. In

the canonical type I IFN signalling pathway, phosphorylated STAT1 and STAT2 molecules then form a heterotrimeric transcription factor (TF) complex together with interferon regulatory factor 9 (IRF9), also known as interferon stimulated gene factor 3 (ISGF3). After entering the nucleus, ISGF3 binds to genomic DNA via the recognition of IFN-stimulated response elements (ISRE) and initiates the transcription of hundreds of so-called interferon-stimulated genes (ISG)[85]. ISGF3 is also the main TF complex formed downstream of the type III IFN receptor, which is composed of the IFNλ receptor 1 (IFNLR1) and the widely expressed IL-10 receptor β chain. As already mentioned above, the restriction of the type III IFN response is limited to mucosal surfaces due to the exclusive expression of IFNLR1 in epithelial cells, hepatocytes and specialized immune cell subsets[86], [87]. It is still a matter of debate to what extent type I and III interferons display functional redundancy at barrier sites.

The IFNγ receptor (IFNGR) is also composed of two subunits, IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2 respectively. In contrast to the other IFNs, IFNγ stimulation leads to the phosphorylation of STAT1 without STAT2, thereby establishing the homodimeric pSTAT1 TF complex γ-activating factor (GAF). GAF binds to γ-activation site (GAS) elements of promoters, agonizing a partially overlapping set of ISGs compared to ISGF3[85]. It is important to note that part of this transcriptional overlap is due to the fact a fraction of phospho-STAT1 also homodimerizes following type I or III IFN receptor stimulation.

Once the transcriptional programs responsible for viral restriction have been initiated, preventing overactivation and subsequent immunopathology becomes important. It is pivotal to understand that most of the proteins exerting regulatory functions are themselves ISGs and thus upregulated in response to IFN stimulation. Phosphatases like Src homology region 2 domain-containing phosphatase (SHP) 1 hydrolyze phosphorylated tyrosine residues on JAKs, STATs and upstream receptors[12], [88]. Additionally, the protein inhibitor of activated STAT (PIAS) 1 has been shown to negatively regulate IFNβ and IFNγ stimulation genes by interfering with the recruitment of phospho-STAT1 to the gene promoter[89]. A third control mechanism is exerted by the SOCS family of proteins. Both SOCS1 and SOCS3 constrain IFN responses by directly suppressing the kinase activity of JAKs and are especially important in early signal desensitization[90]–[92]. Another way to specifically limit IFNAR signalling is via ubiquitin-specific protease 18 (USP18). It has been shown that USP18 specifically binds to the IFNAR2 subunit in a STAT2-dependent manner and inhibits the activity of JAK1 by blocking the interaction between the JAK and the receptor subunit[93], [94].

1.4.3 Susceptibility to severe viral disease

As already outlined above, type I IFNs play a pivotal role in antiviral immunity, which is partly due to their universal activity in almost all nucleated human cells. Hence it is not surprising that all human viral pathogens have developed strategies to evade or subvert the IFN system[85]. A prominent example is Zika virus, a flavivirus responsible for a recent epidemic in South and Central America causing stillbirth and microcephaly in affected infants[95]. Zika virus supresses human type I IFN signalling by its non-structural protein 5 binding to STAT2 ultimately leading to degradation of the latter[96]. Besides virulence factors expressed by viruses, host genetics critically influence disease outcome.

The first monogenic defect of IFN signalling was described in 2003 by Dupuis and co-workers[22]. They reported two children born to consanguineous parents, who died of disseminated viral disease. Targeted sequencing revealed homozygous LOF mutations in the *STAT1* gene in both

patients. Because STAT1 is integral part not only of type I interferon signalling, but affecting all IFN responses, it is not surprising that both patients had previously suffered from disseminated bacillus Calmette-Guerin (BCG) vaccine infection. Antimycobacterial immunity had already been found to be critically dependent on IFNγ since defects in either IFNγ receptor subunit are associated with Mendelian susceptibility to mycobacterial disease[97], [98]. In the following years, various *STAT1* mutations associated with severe and often fatal viral disease caused by CMV, Varicella virus (VZV) vaccine-strain Polio virus or Herpes simplex viruses (HSV) were reported[99]–[102].

Autosomal recessive STAT2 deficiency was identified in a girl with disseminated vaccine strain measles, whose infant brother had already died of an unknown febrile infection[103]. Complete lack of STAT2 expression was found in different family members, of whom some surprisingly reached adulthood without major viral illness. Further studies reported patients with severe disease caused by the measles, mumps and rubella (MMR) vaccine, influenza A virus, enterovirus, respiratory syncytial virus, VZV and Epstein-Barr virus (EBV)[104]–[107]. Deficiency of the third members of the ISGF3 complex, IRF9, has so far only been reported in two families. The first child discovered experienced life-threatening influenza A pneumonia at the age of two years bearing a homozygous truncating *IRF9* variant[108]. In the second family, a homozygous splice variant abrogating IRF9 expression was found responsible for multiple severe viral infections resulting in intensive care unit admissions[109].

Whereas deficiency in one of the ISFG3 components by definition affects more than one IFN family (type I and III in STAT2 and IRF9 deficiency, all three types in STAT1 deficiency), rare variants at the type I IFN receptor level provide proof for the link between type I IFN and susceptibility to viral illness. Homozygous IFNAR2 deficiency led to fatal encephalitis following the inoculation of MMR vaccine[110]. A few years later, IFNAR1 deficiency was described to also result in MMR-induced encephalitis or viscerotropic disease following yellow fever vaccination[111]. Interestingly, all patients deficient for IFNAR subunits were able to handle different viruses including CMV and EBV without major problems prior to vaccination. This raises the question whether patients are protected from viruses encountered via natural routes by compensatory type III IFN activity and potentially specifically susceptible to viruses bypassing this line of defense by injection?

At the level of the receptor-associated kinases downstream of the type I IFN receptor, namely JAK1 and TYK2, observations are complicated by their widespread involvement in multiple different signalling pathways. However, different TYK2-deficient individuals were affected by recurrent HSV infections whereas the only individual described to date carrying biallelic, hypomorphic JAK1 variants mainly suffered from mycobacterial but not viral infection[18], [112], [113].

1.4.4 Type I interferonopathies

If a robust type I IFN response after contact with viral deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) cannot be established, individuals become, as outlined above, susceptible to severe viral disease. This might raise the question whether not only insufficient, but also exaggerated type I IFN activity might be detrimental to human health? In 1984, Aicardi and Goutières reported eight infants suffering from a rapidly progressive and ultimately fatal disorder of the central nervous system (CNS), evident by symmetrical brain calcifications and brain atrophy[114]. The genetic basis of this severe autoinflammatory disease, subsequently termed Aicardi-Goutières syndrome, has been progressively elucidated in recent years. To date, more than 15 monogenic IEIs

have been described to cause CNS and skin inflammation in most cases due to increased IFN activity[115]. The group was therefore named type I interferonopathies. It has become clear that the fundamental requirement to reliably differentiate self-nucleic acids from nonself-nucleic acids is altered in cells of affected individuals. Simply put, this breakdown of self and foreign nucleic acid sensing discrimination results in the misinterpretation of self-DNA and RNA as viral and subsequent excessive type I IFN production[116]. Prime examples are GOF mutations in *IFIH*, *DDX58* encoding retinoic acid-inducible gene I (RIG-I) or *TMEM173* encoding Stimulator of interferon genes (STING)[117]–[119].

Interestingly, not all type I interferonopathies are due to increased type I IFN production. Meuwissen and colleagues reported two families with neonatal onset, severe CNS disease mimicking intrauterine infection. LOF variants in *USP18*, the specific negative regulator of type I interferon signalling described above, were shown to cause increased type I IFN activity underscoring the important and non-redundant role of USP18 in type I IFN regulation[120]. In addition, biallelic loss-of-expression variants in *ISG15* led to a similar phenotype through loss of the stabilising effect of ISG15 upon USP18, allowing excessive ubiquitinylation and subsequent degradation [121].

The aforementioned examples, of how too little type I IFN results in viral susceptibility and too much type I IFN on the other hand produces sterile inflammation, shed light on the importance of fine-tuned signalling pathways in human immunity. The work presented in this thesis aims to add to our understanding of IL-2 and type I IFN signalling in the context of human disease.

2. Materials and Methods

2.1 Primary cells, immortalized cell lines and cytokines

Primary patient or control peripheral blood mononuclear cells (PBMCs) were obtained from whole blood using Ficoll density gradient centrifugation. PBMCs were then washed with phosphate-buffered saline (PBS) and frozen in heat-inactivated fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) at -80°C or liquid nitrogen. After thawing, PBMCs were usually cultured in Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin in the incubator at 37°C with 5% CO_2.

EBV-infected lymphoblastoid cell lines (EBV-LCLs) were generated by incubating 2-5 x 10^6 PBMCs with EBV-containing culture supernatant from a commercially available marmoset B cell line before adding cyclosporin A at a concentration of 1 ug/mL to suppress T cell activation. After seven to fourteen days, small clumps of proliferating B cells became visible under the microscope and could be expanded further. Cells were cultured and frozen as described for PBMCs, only for PhosFlow experiments, EBV-LCLs were cultured in serum-free RPMI medium or X-VIVO 15 medium (Lonza) to reduce basal STAT3 phosphorylation in response to growth factors present in FCS.

Primary fibroblasts were obtained from minced skin biopsies and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% FCS and 1% penicillin/ streptomycin. Cells were passaged every 5-7 days using Trypsin-Ethylenediaminetetraacetic acid (EDTA) (0.05%) to detach cells from the culture flask. For experiments, only fibroblasts passaged fewer than 15 times were used. Cells were frozen as described above and maintained under the same incubator setting as PBMCs.

The erythroleukemia cell line K562, used as a target cell line for NK cells, was purchased from the European Collection of Authenticated Cell Cultures (Cat. No. 89121407) and tested mycoplasma-free. Cells were maintained in RPMI medium supplemented with FCS and antibiotics as stated above.

Primary cells were stimulated using the variety of different cytokines, which are listed in Table 1. Lyophilized cytokines were reconstituted in sterile-filtered PBS containing 0.1% bovine serum albumin (BSA) as a carrier protein and stored in small aliquots at -20°C or -80°C until used.

Cytokine	Concentration	Manufacturer	Code	Figures
IL-2	100 ng/mL	Peprotech	200-02	2, 4, 6, 8, 9, 18
IL-7	100 ng/mL	Peprotech	200-07	2, 4
IL-12	100 ng/mL	Peprotech	200-12	8
IL-15	100 ng/mL	Peprotech	200-15	2, 4, 6, 8, 9
IL-6	100 ng/mL	Peprotech	200-06	18
IL-21	100 ng/mL	Miltenyi Biotec	130-095-768	18
IFNα2b	1,000-10,000 IU/mL	Schering-Plough	00085116801	18, 20, 21-29, 34, 35, 37-41

IFNγ	1,000 IU/mL	Boehringer Ingelheim	L03AB03	20, 21, 36, 38, 40, 41
LPS	100 ng/mL	Sigma-Aldrich	L8274	41
PMA	50 ng/mL	VWR	16561-29-8	13
Ionomycin	100 ng/mL	Calbiochem	56092-82-1	13
IL-3	25 ng/mL	Invitrogen	PHC0033	
M-CSF	100 ng/mL	Invitrogen	PHC9501	
BMP-4	50 ng/mL	Invitrogen	PHC9534	
VEGF	50 ng/mL	Invitrogen	PHC9394	
SCF	20 ng/mL	Miltenyi Biotec	130-096-692	

Table 1. Cytokines.

2.2 Sanger sequencing

Sanger sequencing was performed to confirm variants identified by whole-exome sequencing or to check sequence changes introduced by molecular cloning or gene editing as detailed below. DNA was extracted using the DNeasy or QIAamp DNA mini kit (Qiagen) or the ReliaPrep™ Blood gDNA Miniprep System (Promega) and DNA concentration was checked using Nanodrop (ThermoFisher Scientific). Specific primers were designed in Primer3web version 4.1.0 (https://primer3.ut.ee). Primers were checked for specificity in the University of California Santa-Cruz (UCSC) *in-silico* polymerase chain reaction (PCR) tool (https://genome.ucsc.edu/cgi-bin/hgPcr). Their potential to form homo- or heterodimers was assessed with the help of the OligoAnalyzer™ Tool from IDT (https://eu.idtdna.com/pages/tools/oligoanalyzer). Selected primer sequences were synthesized by Sigma-Aldrich and used at a concentration of 10 uM. The sequences of the primers used can be found in Table 2. For a standard PCR I used the MyTaq HS DNA polymerase (Bioline) according to the manufacturer's instructions, only in case of particularly long fragments the Q5 high-fidelity polymerase (New England Biolabs) was used. Following PCR amplification, the product was purified using the PCR purification kit (Qiagen) and the product length was checked using a 1-2% agarose gel with GelRed nucleic acid stain (Merck) to stain PCR products. DNA fragments of expected length were sent for sequencing with Eurofins Genomics. Sequences were aligned with the consensus coding sequence (human genome assembly 38) in nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene	**Forward primer**	**Reverse primer**
IL2RB	CCTCACAGTGGTTGGCACA	GCACTCTCTCCCTGGGTG
STAT5B c.452T>C	CTCAGTCTTCCTCCCATTCG	ACCAGAGCTGCTTTCCAGTC
STAT5B c.1924A>C	TGGAGATTTTATTGGAGCCATT	ATTCAAGTCTCCCAAGCGGT
IFNAR1	TGTGAGTTTCCTGAGTGTGGA	GGCGTGTTTCCAGACTGTTT
STAT1	TCCCCTGTTGACTTTTCCCC	GCATAGCAAGGACCTGAACC
IRF9	TAGCGGTGCATGCCTGTAG	AGCAAGGACAGAGGGTGAAG

Table 2. Primer sequences.

2.3 Flow cytometry and cell sorting

Flow cytometry was used for various purposes due to its unmatched capacity to reveal information at the single cell level when sample material is limited. In addition to cell-surface immune phenotyping, intracellular proteins, transcription factors as well as signalling intermediates were measured. Furthermore, cell proliferation, killing of target cells, and phagocytic activity were examined using this technology. Before detailing specific assays, some general aspects need to be mentioned: Cells were usually stained at room temperature or 4°C for 30-60 minutes whilst being protected from light. To be able to identify background or non-specific staining, appropriate isotype controls or fluorescence minus one (FMO) controls were used. Compensation was carried out using compensation beads (CompBeads, BD Biosciences or UltraComp eBeads, Invitrogen) in most circumstances, however, cells were used when compensating for viability dyes or cell trace dyes. Cells were washed using PBS containing 2% FCS and 0.1% sodium azide (FACS buffer) to prevent bacterial contamination. Samples were acquired on a Symphony A5 analyzer or a FACS Aria Fusion sorter (both from BD Biosciences). Symphony A5, or FACS Aria Fusion system). The data were analyzed using FlowJo software (Flowjo LLC).

In the *IL2RB* project, where patient material was extremely limited, I combined detailed cell-surface immune phenotyping with a functional assay measuring degranulation and K562 target cell killing in the same tube (see Fig. 6). PBMCs were seeded at 100,000 per well in a 96-well plate and primed with either IL-2 or IL-15 (100 ng/ml each) for 12 hrs or left unprimed. After the priming period, cells were co-incubated with K562 target cells (effector:target ratio of 10:1). In order to be able to identify the K562 within the cell mix, they had been previously labelled with Cell trace violet (Invitrogen) at a concentration of 4 uM. When exposing the labelled K562 to PBMCs, the CD107a-antibody was also added to the wells. Following 3 hrs of co-incubation, cells were harvested, and surface staining was performed for 60 min on ice to inhibit the cytotoxic activity of NK cells. Degranulation was measured by means of CD107a surface expression. Cytotoxicity was assessed by calculating the percentage of 7-AAD-positive K562 after coincubation minus the spontaneous K562 death rate when cultured alone. The addition of IL-2 or IL-15 was found not to affect the viability of K562 cells. Cells were analyzed on the FACSAria Fusion Cell sorter (BD Biosciences) to collect $CD4^+$ and $CD8^+$ T cell as well as NK cells in order to lyse them straight away and extract protein for IL-2Rβ detection by immunoblot.

When analyzing the signalling capacity of the mutant IL-2Rβ protein, I first used PhosFlow, which became part of every project I embarked on during my PhD (see Fig. 2, 4, 18, 23, 24). For this experiment, thawed PMBCs were rested for 4 hrs in serum-free RPMI media. After the addition of surface markers and a fixable viability dye, usually a Zombie dye (Biolegend), to the culture media, 200,000 cells were stimulated for 10 min at 37°C with 100 ng/ml of IL-2, IL-7, or IL-15, or left unstimulated. Cells were then fixed using Cytofix buffer (BD Biosciences) or a home-made fixation buffer containing formaldehyde (ThermoFisher Scientific) at a concentration of 4% in PBS for 20 minutes at 4°C. Following washes, cells were permeabilized using ice-cold PermIII buffer (BD Biosciences) or pure methanol for 20 minutes on ice. Afterwards, extensive washes using FACS buffer were performed to wash away any residual methanol, which would have interfered with intracellular staining. It took my numerous attempts to identify surface markers that resist subsequent methanol treatment or to find clones that recognize their respective epitope after its exposure to methanol. The harsh permeabilization, however, is needed to allow full access to the nucleus where the phosphorylated STAT proteins, in this case pSTAT5, exert their function as transcriptional activators. Following intracellular staining with pSTAT5 for 45 minutes at room temperature, samples were acquired on the Symphony A5 flow cytometer.

Having realized that within the CD4+ T compartment of the IL-2Rβ subject there are some FOXP3^{low} expressing cells (see Fig. 3), I searched for a way to combine FOXP3 and pSTAT5 staining within the same cell. As already mentioned above, commercially available antibodies are not capable of binding FOXP3 after methanol permeabilization which is necessary to get the pSTAT5 signal. Having tested many different fixation and permeabilization methods, I came across the Transcription Factor Phospho Buffer set (BD Biosciences), which conserves transcription factor epitopes by using a two-step permeabilization protocol (see Fig. 4).

When assaying intracellular proteins other than pSTATs, the Cytofix/Cytoperm kit (BD Biosciences) was used as a milder way of permeabilization preserving both epitopes and antibodies, especially tandem-dyes like R-phycoerythrin-cyanine 7 (PE-Cy7). For the detection of intracellular IFNγ (see Fig. 13), 100,000 PBMCs were stimulated with either IL-2, IL-15, or IL-12 for 6 hrs in one experiment or with the immune-dominant CMV peptides pp65 and IE1 at a final concentration of 0.6 nM each (both from Miltenyi Biotec) for 6 hrs with brefeldin A (BD Biosciences) added for the final 5 hrs to prevent secretion of the synthesized IFNγ. In both cases, phorbol 12-myristate 13-acetate (PMA) plus Ionomycin was used as a positive control. Following fixation and permeabilization, staining was performed for 30 minutes at 4°C. To determine the perforin and granzyme B content of NK cells (see Fig. 7) and to assess markers of NK cell maturation (see Fig. 11), again 100,000 PBMCs were stained with the antibodies indicated in Table 3 using the Cytofix/Cytoperm kit and analyzed on the Symphony A5 flow cytometer.

In the STAT5B LOF patient I aimed to assess the T_{reg} compartment both quantitatively and qualitatively. When the patient samples arrived from Bulgaria, PBMCs were extracted as described above and sorted based on CD25 and CD127 expression to obtain pure T_{reg}s. By doing so, their frequency within the CD4$^+$ T cell compartment was compared to different healthy controls including a travel control as well as a freshly taken in-house control (see Fig. 16). The fresh sample had been divided into two parts previously and the second fraction of PBMCs was used to purify CD4$^+$CD25$^-$ effector T cells (T_{eff}). As a first step CD4$^+$ T cells were enriched using RosetteSep (Stemcell Technologies). To isolate CD4$^+$CD25$^-$ T_{eff}, CD25 microbeads and an LD column were used in the MACS® cell separation system from Miltenyi Biotec. Comparing the proliferative capacity of T_{eff}s isolated with this method to sorted T_{eff}s I learned that sorting negatively impacts the proliferation of T_{eff}s. The T_{reg}-depleted, purified T_{eff}s were labelled with Cell trace violet (Invitrogen) at a final concentration of 5 uM and stimulated with anti-CD2/CD3/CD28-human Treg-cell inspector beads (Miltenyi Biotec) for 4 days. The sorted T_{reg} cells from patient, travel control, and fresh in-house control were added at a ratio of 1:1 to the T_{eff} population at the beginning of the stimulation period to assess their capacity to limit T_{eff} proliferation (see Fig. 17).

In the STAT5 GOF project, cell sorting of thawed PBMCs was necessary to assess whether the frequency of the GOF variant was altered in different leucocyte populations after the patient had been treated with Ruxolitinib. Therefore, I established an antibody panel allowing to simultaneously sort T cells (CD3$^+$CD56$^-$), B cells (CD3$^-$CD19$^+$HLA-DR$^+$), NK cells (CD3$^-$CD56$^+$CD16$^+$), Eosinophils (CD3$^-$CD56$^-$CD19$^-$Siglec8$^+$CD193$^+$), and dendritic cells (CD3$^-$CD56$^-$CD19$^-$CD193$^-$CD11c$^+$HLA-DR$^+$). DNA was extracted from sorted populations as stated above and frozen at -80°C. After having established an EBV-LCL from the STAT5B GOF patient, I tried to use single cell sorting to subclone a B cell line where every cell carries the GOF allele for further mechanistic studies. I therefore sorted single, viable (i.e., 7-AAD$^-$) EBV-B cells into 96 well plates at 37°C. The wells contained preconditioned, sterile-filtered RPMI medium containing 20% FCS. However, I failed to grow sufficient cell numbers from these individual cells. PhosFlow on patient PBMCs was carried out as already detailed above using the Cytofix/PermIII buffer combination (see Fig. 18).

During the work on the ISGF3-component deficient cells, I performed flow cytometric analysis of adherent cells for the first time. Induced pluripotent stem cell (iPSC)-derived macrophages were lifted using PBS containing 5 mM EDTA and washed with FACS buffer. Cells were stained with surface markers for 30 minutes at room temperature in the dark (see Fig. 32). When intracellular markers were investigated, cells were fixed using the Cytofix/Cytoperm kit as stated above (see Fig. 40, 41). Intracellular staining was performed for 30 minutes at 4°C in the dark. For the cytokine production assay (see Fig. 41), macrophages were plated at a density of 200,000 cells/mL and preincubated with either IFNα2b or IFNγ at a concentration of 1000 IU/mL for 48 hrs or left untreated. As a second stimulus, LPS was added at a final concentration of 100 ng/mL and cells were further incubated for another 6 hrs. Brefeldin A (Biolegend) was added to allow intracellular accumulation of cytokines under investigation for the last 5 hrs of stimulation. Cells were lifted using 5 mM EDTA and processed as stated above.

To assess the phagocytic activity of the iPSC-derived macrophages 200,000 cells were lifted and placed in Eppendorf tubes containing fresh X-VIVO 15 medium (see Fig. 33). pHrodo Red A Zymosan bioparticles (Invitrogen) were then added at a ratio of 10:1 cells. The negative control was immediately placed on ice, whereas the positive control was put into a shaking incubator for 2 hrs at 37°C. Phagocytosis was terminated by placing the cells on ice. Macrophages were subsequently transferred to FACS tubes and 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific) was added to exclude dead cells. The engulfed bioparticles exposed to pH changes within phagosomes emitted light detectable in the PE-channel (bandpass filter 586±15 nm) of the Symphony A5 flow cytometer.

Antibody	**Fluorochrome**	**Clone**	**Dilution**	**Source**
CD20	BV510	2H7	1:100	Biolegend
PD-1	AF700	EH12.2H7	2:100	Biolegend
CD45RA	BV785	HI100	3:100	Biolegend
CD127	BV605	A019D5	3:100	Biolegend
CD127	PE-Cy7	HIL-7R-M21	3:100	BD Biosciences
TNFα	AF647	MAB11	1:100	Biolegend
CD56	BV711	5.1H11	3:100	Biolegend
CD56	BV421	HCD56	1:100	Biolegend
CD56	PE-CF594	NCAM16.2	1:100	BD Biosciences
CD16	BV650	3G8	1:100	Biolegend
CD19	APC	HIB19	10:100	Biolegend
CD122	PE	TU27	5:100	Biolegend
CD57	BV605	QA17A04	1:100	Biolegend
SYK	APC	4D10.2	3:100	Biolegend
Perforin	PE	dG9	1:100	Biolegend
TCR$\gamma\delta$	FITC	B1.1	5:100	Thermo Fisher Scientific

CD4	BV786	SK3	1:100	BD Biosciences
CD3	BUV395	UCHT1	2:100	BD Biosciences
CD8	BUV496	RPA-T8	1:100	BD Biosciences
CD25	PE	2A3	1:100	BD Biosciences
CD25	BUV737	M-A251	1:100	BD Biosciences
CCR7	PE-Cy7	3D12	3:100	BD Biosciences
CD45R0	APC-H7	UCHL1	3:100	BD Biosciences
Granzyme B	PE-CF594	GB11	1:100	BD Biosciences
IFNγ	AF700	B27	1:100	BD Biosciences
pSTAT5	AF647	47/STAT5	5:100	BD Biosciences
FOXP3	PE-CF594	259D/C7	2:100	BD Biosciences
CD28	BV480	CD28.2	3:100	BD Biosciences
CD95	BV711	DX2	3:100	BD Biosciences
CD107a	BB700	H4A3	5:100	BD Biosciences
CD69	BV421	FN50	1:100	BD Biosciences
IL-2	BV421	5344.111	1:100	BD Biosciences
PLZF	PE-CF594	R17-809	3:100	BD Biosciences
CD132	APC	REA313	3:100	Miltenyi Biotec
FcεRIγ	FITC	Polyclonal	3:100	Merck Milipore
NKG2C	FITC	134591	5:100	R&D Systems
Cell trace violet	NA	NA	1:1000	ThermoFisher Scientific
Zombie aqua	NA	NA	1:200	Biolegend
Zombie NIR	NA	NA	1:100	Biolegend
Zombie UV	NA	NA	1:200	Biolegend
7-AAD	NA	NA	5:100	Biolegend
Live/Dead Fixable Green	NA	NA	1:250	ThermoFisher Scientific
HLA-DR	APC	L243	5:100	BD Biosciences
HLA-DR	BV650	L243	1:100	Biolegend
HLA-DR	FITC	L243	1:10	Biolegend
CD11c	BV421	B-ly6	1:100	BD Biosciences
CD11c	PE-Cy7	3.9	3:100	Biolegend
CD193	APC-Cy7	5E8	5:100	Biolegend
Siglec-8	PE	7C9	5:100	Biolegend

pSTAT1	AF488	4a	5:100	BD Biosciences
pSTAT2	AF647	D3P2P	1:100	Cell Signaling Technology
CD71	APC	CY1G4	3:100	Biolegend
CD206	PE	19.2	5:100	BD Biosciences
CD45	APC-H7	2D1	1:100	BD Biosciences
CD163	BV711	GHI/61	3:100	Biolegend
CD11b	BV785	ICRF44	1:100	Biolegend
CD14	BUV737	M5E2	1:100	BD Biosciences
ICAM1	BV711	HA58	1:100	BD Biosciences
IRF1	PE	20/IRF-1	1:100	BD Biosciences

Table 3. Flow cytometry antibodies. NA: not applicable.

2.4 Immunoblotting

Because of their differences in size and protein content, either 50,000 fibroblasts (see Fig. 19-22) or 1,000,000 EBV-LCLs/PBMCs (see Fig. 25) or 100.000 iPSCs (see Fig. 30) or 50,000 iPSC-derived macrophages (see Fig. 31, 34) were washed in phosphate-buffered saline and lysed on ice in 100 uL lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% Na-Deoxycholate] containing 100 mM dithiothreitol (Sigma-Aldrich), 1× complete protease inhibitor cocktail (Roche), 1x PhosSTOP phosphatase inhibitors (Roche), and 1× NuPAGE Loading Buffer (Life Technologies) for 30 minutes. To shear DNA fragments within the lysates, cells were triturated via a 31-gauge needle (BD Biosciences). Lysates were then heated to 70°C for 10 min before being subjected to 4 to 12% tris-glycine polyacrylamide gel (Novex, Life Technologies) electrophoresis in 1× SDS NuPAGE MOPS Running Buffer (Life Technologies) with Prestained Plus Protein Ladder (ThermoFisher Scientific) as molecular weight markers. Proteins were transferred to 0.45-mm polyvinyl difluoride membranes (ThermoFisher Scientific) in NuPAGE Tris-Glycine Transfer Buffer for 1 hr using a current of 20V. Membranes were blocked for 60 minutes in 5% BSA in tris-buffered saline with 0.1% Tween (TBS-T) before immunostaining by standard methods. A list of anti-human antibodies used together with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies can be found in Table 4. Membranes were washed three times in TBS-T and developed with Immobilon Western Chemiluminescent HRP substrate (Millipore) and imaged on a LI-COR Odyssey Fc (LI-COR).

Antibody	**Host**	**Dilution**	**Source**	**Code**
IL-2Rβ	Rabbit	1:1000	CST	46307
IFNAR1 (N-terminal)	Rabbit	1:200	Abcam	ab124764
IFNAR1 (C-terminal)	Rabbit	1:200	Abcam	ab45172
RSAD2	Rabbit	1:1000	CST	13996
ISG15	Rabbit	1:1000	CST	2743

STAT2	Mouse	1:2000	SCB	sc-1668
pSTAT2	Rabbit	1:2000	CST	8841
STAT1	Rabbit	1:1000	CST	9172
pSTAT1	Rabbit	1:1000	CST	7649
JAK1	Rabbit	1:500	CST	3344
pJAK1	Rabbit	1:500	CST	74129
MX1	Rabbit	1:1000	SCB	sc-50509
α-Tubulin	Mouse	1:10,000	CST	3873
GAPDH	Rabbit	1:10,000	CST	5174
IRF9	Rabbit	1:1000	CST	76684
USP18	Rabbit	1:1000	CST	4813
Anti-rabbit HRP-conjugated	Goat	Various	CST	7074
Anti-mouse HRP-conjugated	Horse	Various	CST	7076

Table 4. Immunoblotting antibodies. CST: Cell Signaling Technology; SCB: Santa Cruz Biotechnology;

2.5 Immunoprecipitation

Roughly 60 million EBV-LCLs were stimulated with IFNα2b for 15 min, washed with ice-cold PBS and lysed in RIPA buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% Na-Deoxycholate, 1 × complete protease inhibitor cocktail (Roche) and 1x PhosSTOP phosphatase inhibitors (Roche)] for 30 minutes. Lysates were centrifuged at 13,000 rpm at 4°C for 10 min. Soluble fractions were pre-cleared for 1 hour at 4°C with Protein G Sepharose 4 Fast Flow beads (GE Healthcare) that had been previously blocked with 1% BSA containing wash buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1x complete protease inhibitor cocktail (Roche, Basel, Switzerland) and 1x PhosSTOP phosphatase inhibitors (Roche)] for 1 hr. Precleared cell lysates were immunoprecipitated overnight with blocked beads that had been incubated with anti-STAT1a antibody or anti-STAT2 antibody for 1 hour and then washed four times in before boiling with 4× lithium dodecyl sulfate buffer at 95°C for 10 min to elute the absorbed immunocomplexes. Immunoblot was carried out as described above with the antibodies listed in Table 5. Since the molecular weight of IRF9 (48 kD) is close to the weight of the heavy chain of the anti-rabbit IgG-HRP conjugate (50 kD), a conformation specific mouse anti-rabbit IgG HRP-conjugate (L27A9) was used as a secondary antibody (see Fig. 27).

Antibody	**Host**	**Dilution**	**Source**	**Code**
STAT1a	Mouse	1:100	SCB	sc-417
STAT2	Rabbit	1:5,000	SCB	sc-476
IRF9	Rabbit	1:1,000	CST	76684

STAT1	Rabbit	1:1,000	CST	9172
STAT2	Mouse	1:2,000	SCB	sc-1668
GAPDH	Rabbit	1:10,000	CST	5174
Anti-rabbit HRP-conjugated	Mouse	Various	CST	5127
Mouse anti-rabbit IgG (conformation specific) HRP-linked	Mouse	1:2,000	CST	3678

Table 5. Coimmunoprecipitation antibodies. CST: Cell Signaling Technology; SCB: Santa Cruz Biotechnology;

2.6 Real-time quantitative PCR

RNA was extracted using the ReliaPrep RNA Cell Miniprep System (Promega) and stored immediately at -80°C if not used straight away. RNA content and quality were assessed using the Nanodrop system (ThermoFisher Scientific) and equal amounts were reverse-transcribed with Superscript III (ThermoFisher Scientific). The resulting cDNA templates were subjected to real-time quantitative PCR (RT-qPCR) with a TaqMan™ Gene Expression Master Mix (Applied Biosystems) according to manufacturer's instructions. Primers and related probes used were designed by Roche Universal ProbeLibrary System Assay Design (Roche) and are included in Table 6. Primers were synthesized by Sigma-Aldrich. Plates were run on an AriaMx Real-time PCR System (Agilent Technologies). The *18S* gene was using as the house-keeping gene and fold gene expression values were calculated using the delta-cycle threshold method (see Fig. 26, 28, 29).

Gene	**Forward sequence**	**Reverse sequence**	**Probe ID**
SOCS1	GCCCCTTCTGTAGGATGGTA	CTGCTGTGGAGACTGCATTG	87
SOCS3	CTTCGACTGCGTGCTCAA	GTAGGTGGCGAGGGGAAG	1
USP18	CAACGTGCCCTTGTTTGTC	ATCAGGTTCCAGAGTTTGAGGT	44
MX1	TGCATTGCAGAAGGTCAGAG	CCTCCATGGAAGAGTCTGTTG	11
RSAD2	GAGGGTGAGAATTGTGGAGAAG	GCGCTCCAAGAATCTTTCAA	9
IFI44L	TGACACTATGGGGCTAGATGG	TTGGTTTACGGGAATTAAACTGAT	15
IRF1	CAGATCTGAAGAACATGGATGC	ACAGGGAATGGCCTGGAT	20
ICAM1	GAAGTGGTGGGGGAGACATA	CCCAATAGGCAGCAAGTTTC	48
CIITA	CAGCTGTGCTCTGGACAGG	TGCTGAGGCTCATGGGATA	80
18S	CCGATTGGATGGTTTAGTGAG	AGTTCGACCGTCTTCTCAGC	81

Table 6. RT-qPCR primer sequences.

2.7 Molecular cloning

In the IFNAR1 project, where patient cells were completely devoid of IFNAR1 expression, I aimed to introduce the wild-type IFNAR1 protein via lentiviral transduction to restore signalling thereby finally proving the IFNAR1-dependence of the in vitro phenotype (see Fig. 22). Searching the non-profit plasmid repository Addgene (https://www.addgene.org/) I came across the destination vector IFNAR1_pCSdest (#53881) which was sent to Newcastle as a kind gift of R. Reeves from Johns Hopkins University, Baltimore. Since I wanted to introduce the IFNAR1 insert into a different destination vector, I had to first clone it into the Gateway® pDONR207 entry vector (ThermoFisher Scientific) using the BP clonase (ThermoFisher Scientific) reaction. After transformation of TOP10 competent cells (ThermoFisher Scientific), I picked several bacterial colonies, expanded them in Luria-Bertani broth (ThermoFisher Scientific) and extracted DNA using the QIAprep Spin Mini Kit (Qiagen). Following this step, I confirmed the successful introduction of the wild-type *IFNAR1* sequence by Sanger-sequencing. Subsequently, I was able to insert the *IFNAR1* cDNA fragment into the pLenti-EF1a-GATEWAY-RSV-Puromycin-GFP (AMS Biotechnology, UK) vector using LR clonase (ThermoFisher). This vector was chosen for different reasons: The elongation factor-1α (EF1a) promotor is a native mammalian promotor displaying strong activity without the risk of getting silenced like CMV-promoters thereby allowing high and persistent expression of the target gene. The puromycin resistance gene under the control of the viral RSV promoter seemed perfect to later on select adherent cells whereas the green fluorescent protein (GFP) gene, controlled by the same promoter, offered the opportunity to quickly assess transfection or transduction efficiency under the microscope.

Lentiviruses were produced by co-transfection of the lentiviral packaging plasmid psPAX2, the envelope plasmid pCMV-VSV-G and the *IFNAR1*-containing lentiviral transfer plasmid in HEK293FT cells (ThermoFisher Scientific) using polyethylenimine (Sigma-Aldrich) as a transfection enhancer. Virus-containing supernatants were harvested at 48 h post-transfection, filtered through 0.45 µm sterile filters and concentrated 100-fold with Lenti-X™ Concentrator (TaKaRa) according to the manufacturer's instructions. Cells were spinoculated in 6-well plates (1.5 hrs, 2000 rpm), with target or null control viral particles in a total volume of 0.5 mL DMEM containing hexadimethrine bromide (Polybrene, 6 mg/mL, Sigma-Aldrich). Cells were rested in virus-containing medium for 4 hrs before incubating in fresh DMEM containing 10% FCS until 48 hrs, when bright GFP expression in the majority of cells was visible under the microscope. The transduced cells were now subjected to selection with 0.75 mg/mL puromycin (Gibco). The appropriate puromycin concentration had been previously determined by a killing curve in untransduced fibroblasts. The antibiotic-containing medium was refreshed every 72 hrs and cells were expanded for subsequent experiments.

2.8 iPSC maintenance and gene editing

The parental iPSC line (SFC856-03-04) used for clustered regularly interspaced short palindromic repeats (CRISPR) editing, generated from a healthy adult donor at the University of Oxford, has been described previously[122], is registered in the Human Pluripotent Stem Cell Registry (https://hpscreg.eu/) and is available from the European Bank for induced pluripotent Stem Cells (https://ebisc.org/). The iPSC cells were cultured using mTeSR1 medium (Stemcell Technologies) in 6 well plates coated with the growth factor basement membrane matrix Geltrex (Life Technologies). The medium was replaced every day and the absence of differentiating cells was confirmed under the microscope.

For gene editing the Alt-R CRISPR-Cas9 system (IDT) was used aiming to induce gene deletions that remove exon-intron boundaries thereby creating a frameshift which subsequently ablates gene expression through nonsense mediated mRNA decay. To induce the desired out-of-frame deletions, I decided to use two different guide-RNAs (gRNA) at a time with at least one of the two targeting an intronic region between exons 3 to 5 of both *STAT1* and *IRF9*. iPSCs with a *STAT2*-gene disruption had already been created by fellow group-member Dr. Christopher Duncan. The strategy of targeting exons 3-5 was chosen since a deletion further upstream could potentially result in the activation of an alternative start site leading to expression of a truncated protein. To introduce guide RNAs alongside a tracrRNA as well as the Cas9 enzyme into the iPSCs, the Neon transfection system (ThermoFisher Scientific) was used. The efficiency of the different gRNA pairs in inducing a deletion was compared in transfected iPSC by running the PCR product on an agarose gel and only the iPSCs transfected with most effective pair of gRNAs were taken forward. The sequences of the gRNAs used are listed in Table 7.

Gene	**Guide RNA sequence**
STAT1	GAGGUCAUGAAAACGGAUGG
STAT1	GCUUUUAGCAGCAGUUUAUG
IRF9	CAGCAACUGAUACACCUUGU
IRF9	GAGCUCAGAAGGGAUUAUGC

Table 7. Guide RNA sequences.

After transfection of the iPSC pool, cells were plated at low density on irradiated mouse embryonic fibroblasts to allow single cell colonies to grow. Individual colonies were picked after 7 days under the microscope and expanded in 96 well plates. After my first attempt of picking individual colonies under the microscope placed in the tissue culture hood, bacterial contamination of the well occurred. I therefore added the penicillin/streptomycin mix the day before picking colonies for the second try. Additionally, I wore a face mask and extra-long, sterile gloves to circumvent another contamination. The picked colonies I then expanded in Geltrex-coated 96 well plates using the p160-Rho-associated, coiled-coil containing protein kinase (ROCK)-inhibitor (Y-27632, Abcam) to increase survival of the feeder-deprived, detached iPSCs. Growing colonies were expanded into 24 well plates, with a fraction of the cells being lysed for DNA extraction to screen colonies and to limit the number of clones in culture. DNA was extracted in the 96 well plates using a home-made lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA, 10 mM NaCl, 0.25% Triton X100, 1mg/ml proteinase K (Sigma Aldrich)], with which cells were incubated overnight at 55°C. DNA was subsequently precipitated using ice-cold ethanol containing 75 mM NaCl and left to air-dry. Using the PCR primers detailed in Table 2 and running to PCR products on agarose gels, clones with potentially successful gene disruption could be identified. This screening method was particularly important in the case of IRF9 since this protein is not expressed in iPSCs. To confirm STAT1 and STAT2 loss-of-expression variants, immunoblot was carried out as described above.

2.9 iPSC-derived macrophage differentiation

The differentiation process was carried out following the protocol published by van Wilgenburg et al.[123]. The gene-edited iPSCs were seeded on AggreWell 800 plates (Stemcell Technologies) at a density of 4 million cells/mL. This concentration of cells is crucial not to risk spill-over from

one well to another. Cells were gently centrifuged into the wells at 100 g for 3 minutes. Embryoid bodies were carefully fed every day with mTeSR medium containing bone morphogenetic protein 4 (BMP-4, 50 ng/mL), vascular endothelial growth factor (VEGF, 50 ng/mL) and stem cell factor (SCF, 20 ng/mL). They were harvested at day 4 by gently pipetting up and down using a 5 mL serological pipette and transferred into T175 flasks containing X-VIVO 15 medium (Lonza), supplemented with macrophage colony stimulating factor (M-CSF, 100 ng/mL) and IL-3 (25 ng/mL, both from Invitrogen). After several weeks with weekly media changes, macrophage precursors could be harvested. These were plated at a density 200,000 cells/mL and terminally differentiated in X-VIVO 15 medium containing M-CSF in the abovementioned concentration. After 7 days, macrophages were ready to be used for experiments. To confirm that the differentiated cells express key features of macrophages, analysis of different cell surface markers was assessed by flow cytometry (see Fig. 32) and a phagocytosis assay (see Fig. 33) was carried out as described below. Additionally, STAT1, STAT2 and IRF9 were assessed by immunoblot to ensure knock-out of the specific protein (see Fig. 31).

2.10 Targeted transcriptomics

RNA was extracted by lysing 200,000 iPSC-derived macrophages stimulated with either IFNα2b or IFNγ for 1 or 48 hrs in TRIzol reagent (ThermoFisher Scientific) and stored at -80°C until use. RNA was purified using the RNA Clean and Concentrator-5 kit (Zymo Research) before 100 ng of total RNA were loaded onto the Nanostring nCounter cartridge following the manufacturer's instructions. Raw data files were imported into the nSolver 4.0 software. All samples passed quality control assessment. Data normalization included positive control normalization as well as mRNA content normalization based on the expression of the housekeeping genes *ABCF1*, *GUSB*, *MRPS7*, *NMT1*, *NRDE2*, *OAZ1*, *PGK1*, *SDHA* and *TBP*. Log2-transformed expression or ratio data were exported and visualized using GraphPad Prism version 9.0.2 (GraphPad Software) (see Fig. 35-38). Differentially expressed genes were analyzed for overrepresented conserved transcription factor binding sites in the promoter sequence (±10,000 bases) by single site analysis using the oPOSSUM database 3.0[124] with a conservation cutoff of 0.4 and matrix score threshold of 85%. Promoters were examined for the presence of ISGF3 (ISRE), IRF1, and STAT1 (GAS) binding sites (see Fig. 39).

2.11 Statistical analysis

Whenever sufficient patient material was available, experiments were repeated a minimum of three times. Data were mostly normalized/log10-transformed before parametric tests of significance were performed in light of the limitations of ascertaining distribution in small sample sizes and the high type II error rates of nonparametric tests in this context[125]. Comparisons of two groups were done by student *t* test. Comparisons of more than two group used one-way analysis of variance (ANOVA) with post-test correction for multiple comparisons or the non-parametric Kruskal Wallis-test in the T_{reg} suppression assay. All statistical analyses were performed using GraphPad Prism (v9.0.2). All tests were two-tailed with with $\alpha \leq 0.05$.

3. Results

3.1 IL2RB deficiency as a new T_{reg}opathy

3.1.1 Case report and genetics

We studied a girl born to consanguineous parents of Pakistani origin at 37 weeks of gestation with intrauterine growth retardation (Patient 1). Dry skin and loose stools were noted early on and progressed to severe secretory diarrhea, leading to her emergency readmission to hospital in hypovolemic shock at four weeks of age. At this time, Patient 1 presented with severe metabolic acidosis and her weight was only 1,850g. She received intravenous fluid resuscitation in the emergency department and subsequent total parenteral nutrition to allow her bowel to recover. Endoscopic evaluation of the upper digestive tract showed severe architectural distortion and effacement of duodenal villi, associated with chronic inflammatory infiltration and marked apoptosis of epithelial cells. Biopsy of an associated skin rash revealed inflammatory changes suggestive of acrodermatitis enteropathica, although serum zinc levels were normal.

In line with a potential autoimmune genesis, Patient 1 developed a positive direct antiglobulin test and moderate thrombocytopenia alongside hypergammaglobulinaemia and positive anti-nuclear and smooth-muscle autoantibodies. Inflammatory markers were raised, and she required respiratory support, but criteria for HLH were never fulfilled. She developed hepatitis associated with CMV viremia that was successfully treated with ganciclovir. At this time her peripheral blood immunophenotype revealed normal numbers of T cells with a reduced CD4/CD8 ratio and relatively low naive T cell percentage. In whole blood assays, poor production of IL-10 was noted as well as overall poor T cell cytokine responses that were not improved by the addition of IL-2. T cell proliferation responses were globally reduced and again displayed insensitivity to exogeneous IL-2. Regarding the B cell compartment, an increased proportion of class-switched memory B cells was found in accordance with the serum hypergammaglobulinaemia.

Patient 1 was immunosuppressed using steroids, anti-tumour necrosis factor α (TNFα) therapy with infliximab, and sirolimus with significant improvement in her diarrhea, dermatitis, and nutritional status. However, she could not be weaned from parental nutrition and experienced periods of presumed inflammatory pneumonitis and Staphylococcus aureus sepsis. Owing to the severity of her immune dysregulation and the suspicion of a monogenic etiology, she underwent peripheral blood haematopoietic stem cell transplantation (HSCT). Following prompt engraftment, she reactivated CMV despite anti-infective prophylaxis and developed severe pneumonitis culminating in respiratory failure and her death at the age of eleven months[126].

Given the consanguineous background of the family an autosomal-recessive mode of inheritance was hypothesized and whole exome-sequencing (WES) was carried out in Patient 1 as well as her parents. In the *IL2RB* gene, we found a nucleotide substitution (A>G) on position 230 in exon 3 thereby replacing a conserved leucine residue by a proline (p.L77P) in the N-terminal fibronectin-III domain. While Patient 1 was homozygous for this variant, both parents were found to be heterozygous. The variant was confirmed by Sanger sequencing and had not been reported in a homozygous manner in various publicly available databases including gnomAD and ExAC. A scaled combined annotation-dependent depletion (CADD) score of 13.17 further suggested a functional impact of the variant[127].

3.1.2 Reduced IL-2Rβ expression and downstream signalling

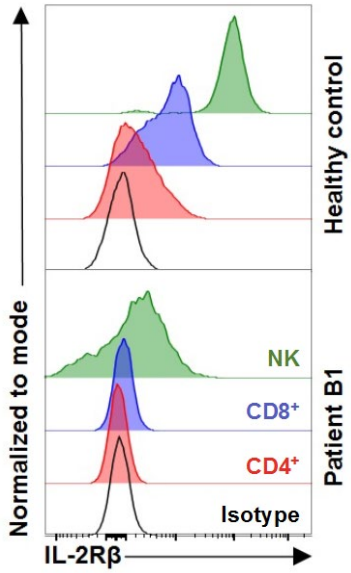

Figure 1. **Reduced IL-2Rβ expression in Patient 1**. NK cells (green), CD8+ (blue) and CD4+ (red) T cells, Isotype control in black. Repeat experiments n=3. With permission from Rockefeller University Press.

To assess whether the *IL2RB* variant found in Patient 1 affects protein expression, we analyzed IL-2Rβ expression on the cell surface of different lymphocyte subsets via flow cytometry (Fig. 1). In healthy control cells, the highest IL-2Rβ expression was found on NK cells, followed by $CD8^+$ T cells; CD4+ T cells displayed less than their cytotoxic counterparts whereas B cells had almost no IL-2Rβ on their surface. In primary cells from Patient 1, we noted a severe reduction of IL-2RB surface expression to 1-3% consistently across cell types with substantial residual expression detected only on NK cells.

The capacity of the residual surface $IL\text{-}2R\beta^{L77P}$ to induce downstream-signalling was addressed by studying phosphorylation of STAT5 in response to stimulation with IL-2, IL-15 or IL-7 (Fig. 2). A severe reduction of STAT5 phosphorylation after both IL-2 and IL-15 stimulation was obvious in T cells from Patient 1, especially in the $CD4^+$ compartment, whereas patient NK cells, in line with their residual IL-2Rβ expression, were found to phosphorylate STAT5 comparably to healthy controls, at least at high cytokine concentrations. In contrast, we observed consistently elevated pSTAT5 levels in T cells of Patient 1 after stimulation with IL-7 thereby firstly proving the integrity of the JAK-STAT-signalling pathway in our patient and secondly raising the possibility of secondary effects in the IL-2/IL-15 signal deprived T cells.

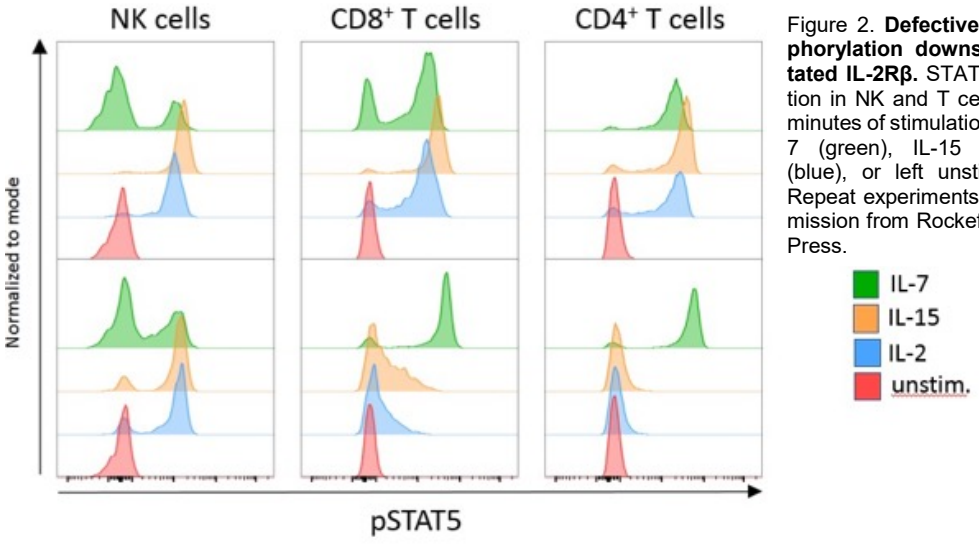

Figure 2. **Defective STAT5 phosphorylation downstream of mutated IL-2Rβ.** STAT5 phosphorylation in NK and T cells following 10 minutes of stimulation with either IL-7 (green), IL-15 (orange), IL-2 (blue), or left unstimulated (red). Repeat experiments n=3. With permission from Rockefeller University Press.

3.1.3 T_{reg} deficiency causing autoimmunity

The striking autoimmune phenotype seen in Patient 1 and the reduced T_{reg} numbers present in *Il2rb*$^{-/-}$ mice prompted me to investigate the patient's T_{reg} compartment. We found severely reduced numbers of $CD25^{high}$ $FOXP3^+$ T_{reg}s compared to healthy control cells. (Fig. 3). Interestingly, we noted an unusual population of $CD25^{neg}$ $FoxP3^{low}$ cells in the $CD4^+$ T cell compartment of Patient 1, absent from controls. The occurrence of such a population had also been described in a *Il2rb*$^{-/-}$ mouse model and found to be IL-7 receptor dependent. However, these cells failed to

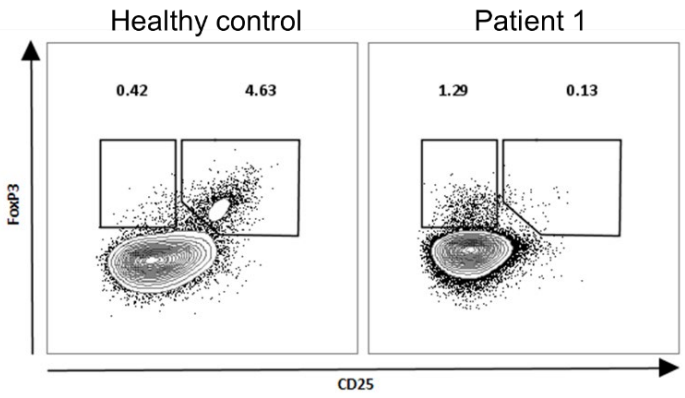

Figure 3. **Lack of classical T_{reg}s in Patient 1**. Gated on $CD3^+$ $CD4^+$ cells. Repeat experiments n=3. With permission from Rockefeller University Press.

control autoimmune disease in mice[128]. Due to the very limited size of these populations in Patient 1 I was unable to investigate the function of residual T_{reg}s in a suppression assay. However, looking at STAT5 phosphorylation of both these subsets, a reduced but not absent response was noted (Fig. 4). Taken together, the profound reduction of STAT5 signalling within the $CD4^+$ T cell compartment and the absence of $CD25^{high}$ $FOXP3^+$ T_{reg}s closely mirrors the situation in *Il2rb*$^{-/-}$ mice and other known human T_{reg}opathies such as deficiency states of FOXP3 and CD25. Therefore, this could be sufficient to explain the various autoimmune manifestations observed early in the life of Patient 1.

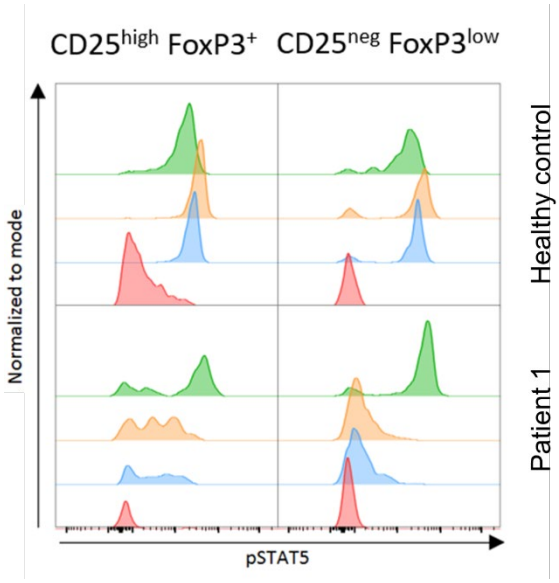

Figure 4. **Reduced STAT5 phosphorylation in T_{reg} populations.** Short-term stimulation with IL-7 (green), IL-15 (orange), or IL-2 (blue) stimulation. Unstimulated cells are shown in red. Gates according to Fig. 3. Data representative of 3 independent experiments.

3.1.4 NK cell abnormalities and the failure to control CMV

Since Patient 1 had suffered from CMV-triggered hepatitis and had succumbed to CMV pneumonitis post-HSCT I was interested in studying the lymphocyte populations important for the immune response to CMV, namely NK cells and $CD8^+$ T cells. When checking IL-2Rβ expression initially, I had noted normal numbers of $CD3^-$ $CD56^+$ NK cells in the patient. This was different from *Il2rb*$^{-/-}$ mice, where a profound defect of NK cell development had been reported earlier[129]: in addition to the reduced number of peripheral NK cells, their capacity to kill target cells as well as to produce IFNγ had been found nearly absent[129]. When assessing NK cell subpopulations of Patient 1 an expansion of the more

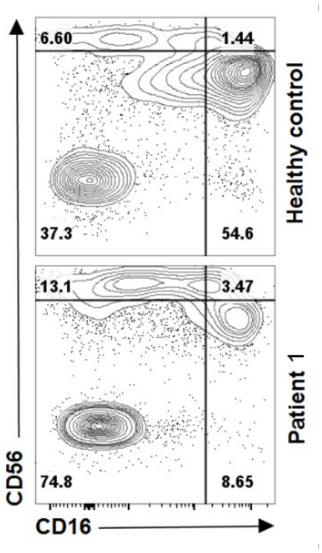

Figure 5. **Expansion of $CD56^{bright}$ NK cells in Patient 1.** Gated on $CD3^-$ $CD19^-$ lymphocytes. Repeat experiments n=4. With permission of Rockefeller University press.

immature CD56^{bright} NK cells accounting for approximately 50% of all NK cells became apparent (Fig. 5). To assess their functional capacities, I set up a flow cytometry-based assay combining degranulation via CD107a upregulation and killing of fluorescently labelled K562 target cells. The

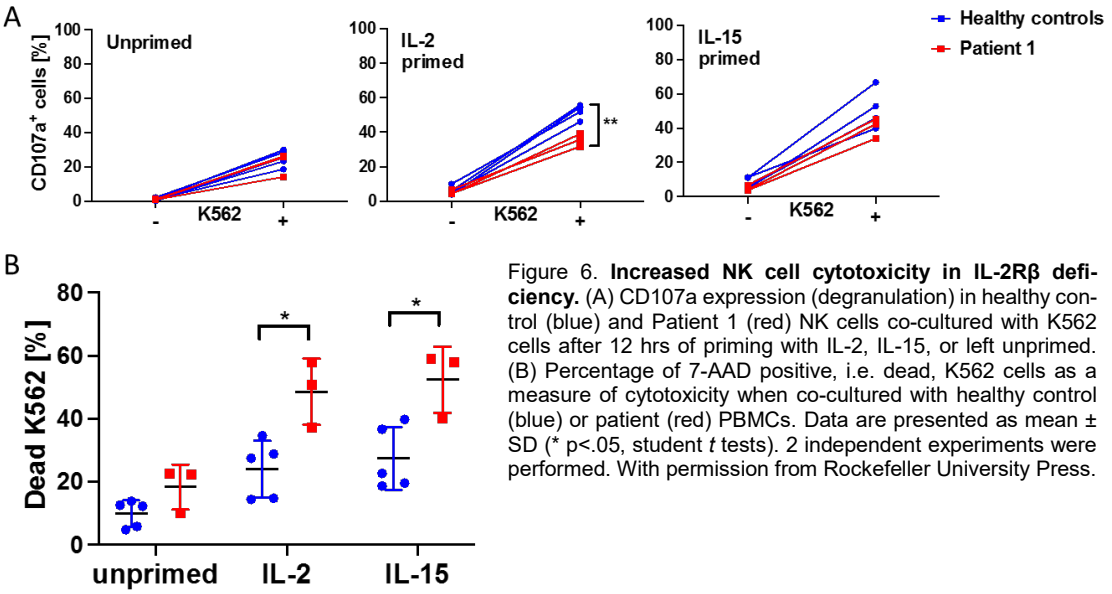

Figure 6. **Increased NK cell cytotoxicity in IL-2Rβ deficiency.** (A) CD107a expression (degranulation) in healthy control (blue) and Patient 1 (red) NK cells co-cultured with K562 cells after 12 hrs of priming with IL-2, IL-15, or left unprimed. (B) Percentage of 7-AAD positive, i.e. dead, K562 cells as a measure of cytotoxicity when co-cultured with healthy control (blue) or patient (red) PBMCs. Data are presented as mean ± SD (* p<.05, student *t* tests). 2 independent experiments were performed. With permission from Rockefeller University Press.

cells were additionally primed with either IL-2 or IL-15 for 12 hours or left unprimed. I was very surprised to see that NK cells of Patient 1, despite a comparable or in the case of IL-2 priming reduced degranulation (Fig. 6A), killed more K562 target cells than different healthy control cells (Fig. 6B). This trend was present under baseline conditions and became statistically significant following priming with IL-2 or IL-15. Whereas I had expected a failure of NK cell cytotoxicity in keeping with the clinically observed susceptibility for CMV complications, I was confronted with enhanced target cell killing of IL2Rβ^{L77P} NK cells.

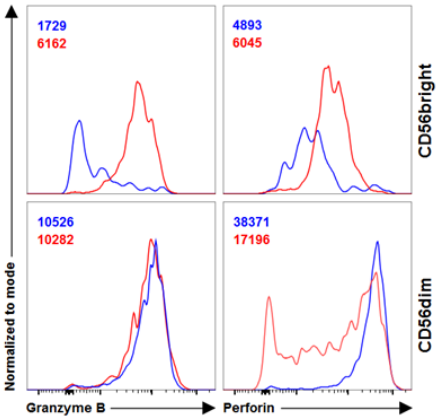

Figure 7. **Increased Granzyme B and Perforin content in CD56^{bright} NK cells from Patient 1.** Numbers indicate MFIs of patient 1 (red) and healthy control (blue) cells. Representative histograms from 3 independent experiments are shown. With permission from Rockefeller University Press.

After going back to the literature, I realized that an expansion of the CD56^{bright} NK cell compartment with enhanced *ex vivo* cytotoxicity had previously been reported in humans experiencing low-dose IL-2 treatment[130]–[134], CD25 blockade[135], [136] or IL-15 therapy[137] for malignant or autoimmune diseases. In the context of Patient 1, a similar situation was likely to pertain because of the selective preservation of IL-2Rβ expression by NK cells in a context where IL-2 and IL-15 are produced normally but not consumed due to the lack of sufficient IL-2Rβ on the surface of T cells. Thus, responsiveness to IL-2 and IL15, albeit at high concentrations, was preserved in IL-2Rβ^{L77P} NK cells and their cytotoxic function was enhanced in vitro, in keeping with chronic *in vivo* stimulation and/or reduced Treg suppressor activity. With the help of Dr. Rainer Döffinger from the University of Cambridge, we were able to confirm elevated concentrations of both cytokines in the serum of Patient 1. Additionally, I found increased expression of cytotoxic effector molecules Perforin and Granzyme B in CD56^{bright} NK cells from Patient 1 (Fig. 7). However, not

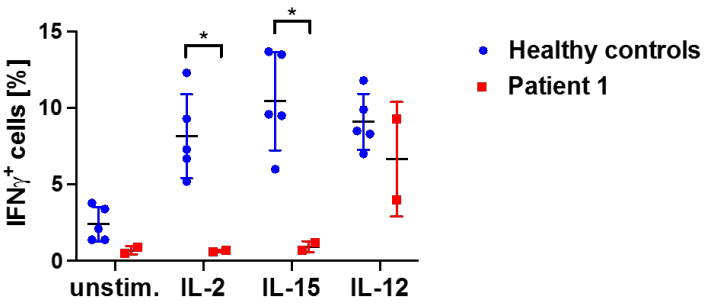

Figure 8. **Defective IFNγ production in response to IL-2 and IL-15.** Representative of n=2 experiments. Data are displayed as mean ± SD (*, $p < 0.05$; Student *t* tests). With permission from Rockefeller University Press.

all effector functions of IL2Rβ^{L77P} NK cells were enhanced by the chronic IL-2 and IL-15 stimulation. IFNγ-production in response to IL-2 and IL-15 was nearly absent (Fig. 8) and CD25 upregulation was found reduced (Fig. 9).

At first sight these findings appeared at odds with the susceptibility to CMV disease seen in Patient 1. However, a more detailed examination of the NK compartment revealed a lack of the terminally differentiated CD57$^+$ NK cells that have been shown to mature from CD57$^-$CD56^{dim} NK cells in response to IL-2 *in vitro*[138] or the presence of CMV *in vivo*[139] (Fig. 10, red gate). In particular, Patient 1 lacked the memory-like NK cells defined by the co-expression of CD57 and killer cell lectin like receptor C2, also known as KLRC2 (or NKG2C), which typically accumulate in the context of CMV infection in healthy children and adults. However, evidence for the importance of this latter subset in controlling CMV infection is sparse and individuals harbouring a deletion of the NKG2C gene handle CMV infection normally[140]. To explore whether there was a general problem with NK maturation, I examined the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) and its downstream targets spleen tyrosine kinase (SYK) and the high affinity immunoglobulin ε receptor subunit γ (FcεRIγ)[141], [142]. This analysis confirmed a failure of NKG2C$^+$ NK cells from Patient 1 to complete their differentiation by downregulating PLZF, SYK, and FcεRIγ, as well as upregulating CD57 (Fig. 11). The clinically observed CMV susceptibility might hence be explained at least in part by a NK cell maturation defect occurring in the context of the hypomorphic *IL2RB* mutation p.L77P.

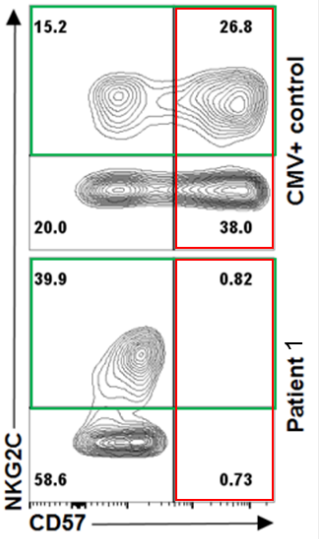

Figure 10. **Absent CD57$^+$ memory NK cells in Patient 1.** The green gate comprises NCG2C-positive cells, whereas the red marks CD57-positive cells. Representative flow plots from 3 independent experiments are shown. With permission from Rockefeller University press.

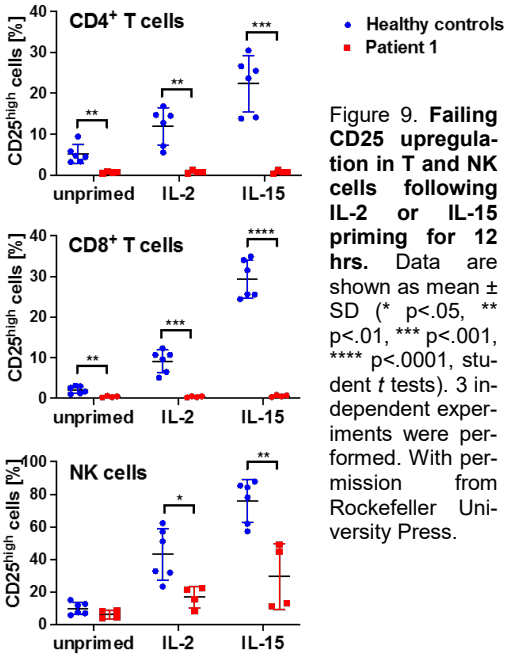

Figure 9. **Failing CD25 upregulation in T and NK cells following IL-2 or IL-15 priming for 12 hrs.** Data are shown as mean ± SD (* p<.05, ** p<.01, *** p<.001, **** p<.0001, student *t* tests). 3 independent experiments were performed. With permission from Rockefeller University Press.

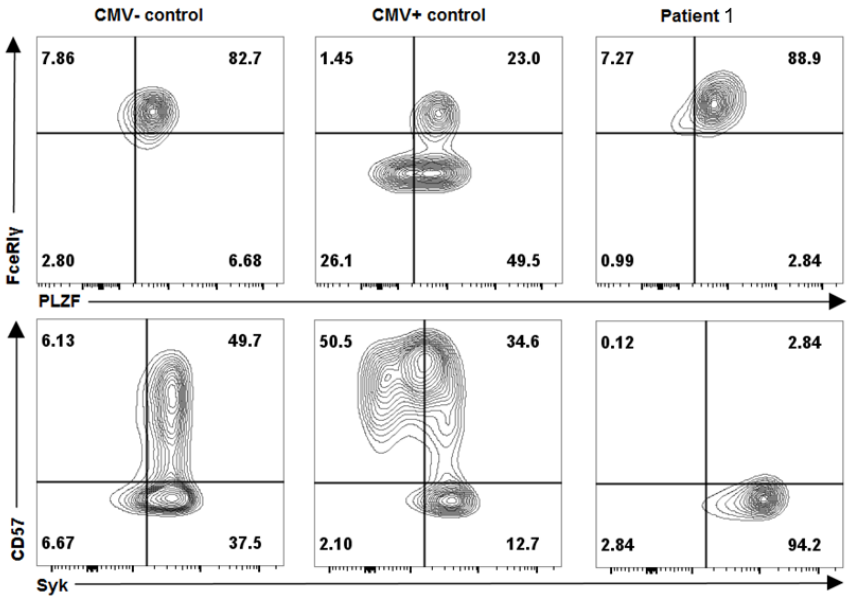

Figure 11. **Defective NK cell memory formation in Patient 1.** FcεRIγ, PLZF, and SYK expression in CMV⁻ and CMV⁺ healthy controls and well as the patient, gated on NKG2C-expressing NK cells (green gate in Fig. 10). Representative histograms from 3 independent experiments are shown. With permission from Rockefeller University Press.

3.1.5 Cytotoxic T cell alterations

The other lymphocyte subset crucial for mounting an effective immune response to CMV is cytotoxic T cells. In contrast to other herpesviruses, CMV infection induces a population of atypical CD8⁺ memory T cells, which does not contract once the primary lytic infection has been cleared;

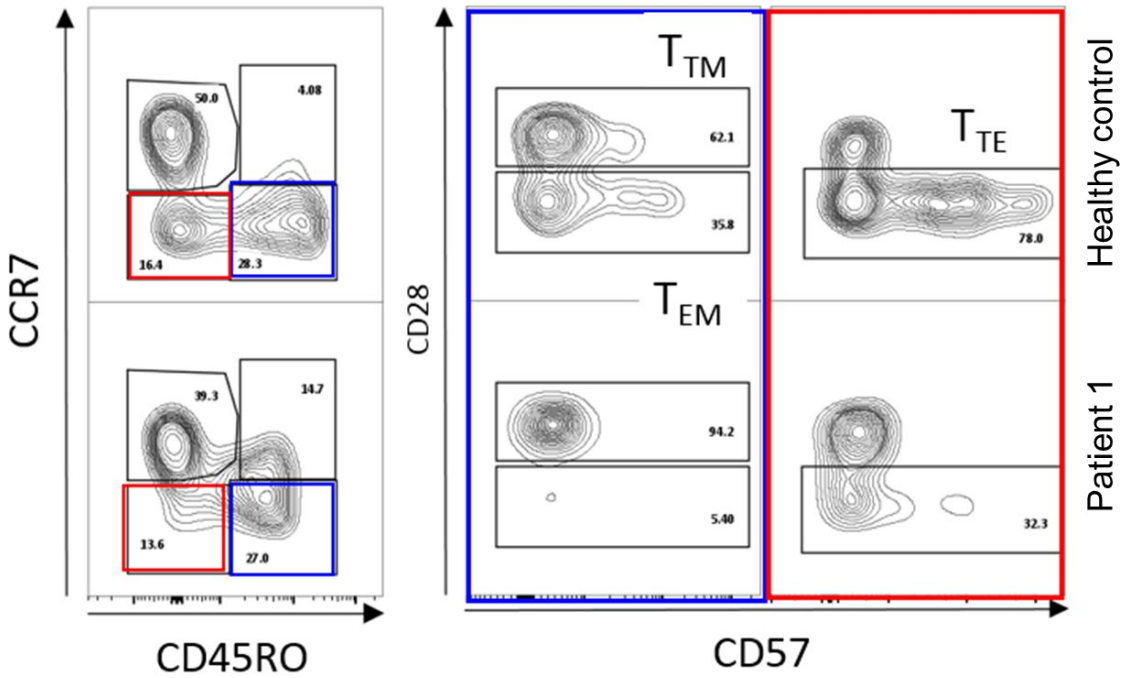

Figure 12. **Lack of CD8⁺ T_{EM} cells in Patient 1.** Representative flow plots from 3 independent experiments are shown. T_{TM}: Transitional memory T cells; T_{EM}: Effector memory T cells; T_{TE}: Terminal effector T cells.

this phenomenon has been termed 'memory inflation'[143]. These T cells show an effector memory phenotype (T_{EM}) with downregulation of costimulatory receptors like CD28, expression of the terminal differentiation marker CD57 and high levels of perforin and granzyme B. When I investigated CD8 memory populations in Patient 1, a striking lack of T_{EM} cells was noted despite the proven CMV infection (Fig. 12). Additionally, few terminal effector cells (T_{EMRA}) and very little CD57 expression were seen, thereby closely mirroring the observation in NK cells of Patient 1.

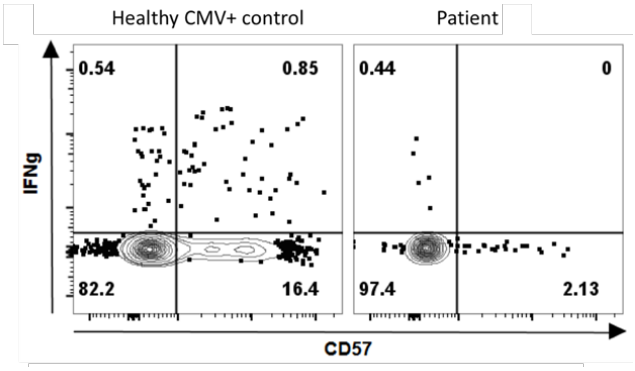

Figure 13. **Missing CMV-specific T cells in Patient 1**. Gated on CD8⁺ T cells. Representative flow plots from 2 independent experiments are displayed. IFNg: IFNγ.

Moreover, when stimulating T cells of Patient 1 with peptide pools containing the immunodominant CMV-proteins pp65 and IE-, IFNγ-producing, CMV-specific memory CD8⁺ T cells were completely missing (Fig. 13). Taken together, the proband's T cells seem to recognize CMV peptides, and start memory formation, but memory inflation of CD8⁺ T_{EM} cells is failing. Since only T_{EM} and terminal effector T cells (T_{TE}) cells carry substantial amounts of cytolytic granules like Perforin and Granzyme B (Fig. 14), difficulties to efficiently kill CMV-infected cells might be anticipated in IL2Rβ-deficient T cells.

A potential explanation for these observations again involves IL-7: during normal CD8⁺ T cell maturation, surface expression of IL-2Rβ increases (Fig. 15), most likely reflecting an increased dependence on IL-15 survival signals[144]. IL-7 signaling on the other hand drives early T cell development as demonstrated by the lack of T cells in patients with IL-7 receptor α deficiency[145], [146]. However, during terminal T cell maturation, the IL-7 receptor is downregulated and T_{EM} and T_{TE} usually display very low or absent surface expression thereof. In light of the heightened IL-7 signal strength observed in T cells from Patient 1 (Fig. 2) it seems plausible that T cells with IL-2Rβ^{L77P} are sustained by IL-7 signals only as long as their differentiation program allows expression of the IL-7 receptor. The corresponding failure to establish a long-lived and antigen-experienced, CMV-specific cytotoxic T cell population is an attractive mechanism that might again contribute to the specific CMV susceptibility seen in Patient 1.

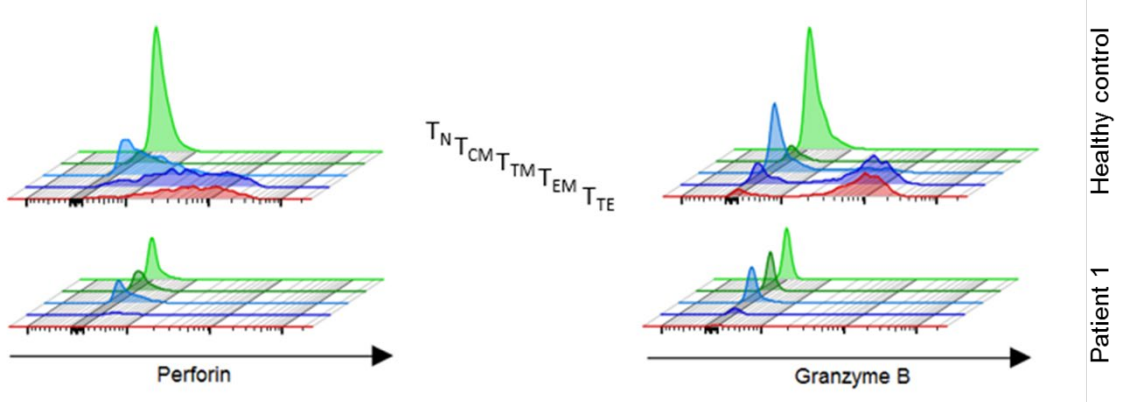

Figure 14. **Failure to accumulate cytotoxic effector molecules during T cell maturation in Patient 1.** Representative flow plots from 2 independent experiments. T_N: Naive T cells; T_{CM}: Central memory T cells; T_{TM}: Transitional memory T cells; T_{EM}: Effector memory T cells; T_{TE}: Terminal effector T cells.

3.1.6 Identification of additional IL2RB-deficient patients

After I had presented the data on Patient 1 at the annual meeting of the European Society for Immunodeficiencies, Dr. Mike Lenardo from the National Institute of Health offered a collaboration on this project. His lab had identified two siblings harbouring the same homozygous *IL2RB* p.L77P variant with a very similar phenotype. His PhD-student Zinan Zhang identified sequestration of the IL2Rβ^{L77P} variant in the endoplasmic reticulum as the underlying reason for the reduced surface expression using an overexpression system[126]. Two additional *IL2RB* alleles, namely p.S40L and p.Q96*, which had also been identified in additional families by their collaborators,

could only be studied in recombinant systems *in vitro* since all affected patients had already succumbed to disease and no primary patient material was available. The definite proof that *IL2RB* variants are causative of the observed IL-2 signalling defect was established by overexpressing wild type-*IL2RB* in patient T cells using lentiviral transduction. Zinan Zhang showed that IL-2 responsiveness of patient IL2Rβ^{L77P} cells could be partially rescued by the introduction of the wild type allele thereby demonstrating causality[126]. Another family in which the homozygous *IL2RB* variant p.P222_Q225del caused immune dysregulation in two siblings was reported in the same issue of the *Journal of Experimental Medicine,* further substantiating the evidence that biallelic variants in *IL2RB* constitute a new IEI associated with severe autoimmunity and CMV disease[147].

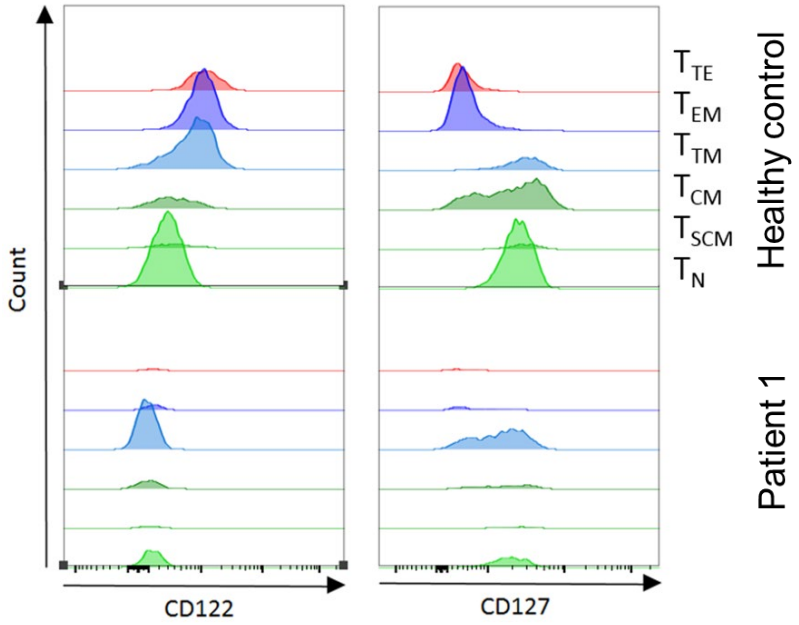

Figure 15. **Lack of CD127^{low} cytotoxic memory T cell populations in Patient 1.** IL2Rβ (CD122) and IL7Rα (CD127) expression in different CD8$^+$ T cell memory populations. Histograms represent 2 independent experiments. T_N: Naïve T cells; T_{SCM}: Stem cell memory T cells; T_{CM}: Central memory T cells; T_{TM}: Transitional memory T cells; T_{EM}: Effector memory T cells; T_{TE}: Terminal effector T cells.

3.2 T_{reg} dysfunction in STAT5 LOF disease

3.2.1 STAT5B deficiency

Human STAT5B deficiency was first described by pediatric endocrinologists caring for a girl with short stature and insensitivity to growth hormone therapy in 2003[66]. Postnatal growth failure as a result of insufficient growth hormone receptor signal transmission remained the characteristic symptom as more patients were diagnosed[148], [149]. Interestingly, both homozygous as well as heterozygous variants acting in a dominant negative fashion were experimentally shown to impair STAT5 function. The majority of LOF variants are located in the coiled-coil or the DNA-binding domains[150]. Additional symptoms of immune dysregulation like severe eczema, chronic lung disease with the histologic pattern of lymphoid interstitial pneumonia or various autoimmune manifestations have been noted subsequently[150]–[152]. Immunologic abnormalities included hypergammaglobulinaemia, T cell lymphopenia affecting both CD8$^+$ and CD4$^+$ subsets, albeit with a particular reduction of T_{reg}s.

3.2.2 Clinical and genetic evaluation of a new patient

A male adolescent from Bulgaria (Patient 2) was referred to the Pediatric Immunology Service in Newcastle because of severe atopic dermatitis, autoimmune thyroid disease and enteropathy as

well as autoimmune alopecia. He had been born to consanguineous parents and presented with growth hormone-insensitive growth failure in early childhood. Intermittent systemic prednisolone therapy and cyclosporin were used with limited success. During the work-up it became clear that a chronic and progressive lung disease with exercise intolerance was also part of his disease, however, a lung biopsy was not performed. Whole exome sequencing identified a homozygous missense variant in STAT5B leading to amino acid exchange from leucine to proline on position 151 (p.L151P). At this time, only two pathogenic homozygous missense variants had been identified in *STAT5B*, both located in the SH2 domain and interfering with regular protein folding[153], [154]. To assess the potential pathogenicity of the variant, my colleague Meghan Acres tested STAT5B expression in EBV-LCLs and found it to be expressed at similar levels compared to wild type cells[155]. However, when analyzing STAT5 phosphorylation in response to IL-2, she identified a reduced signalling capacity indicating a hypomorphic behaviour of the p.L151P allele.

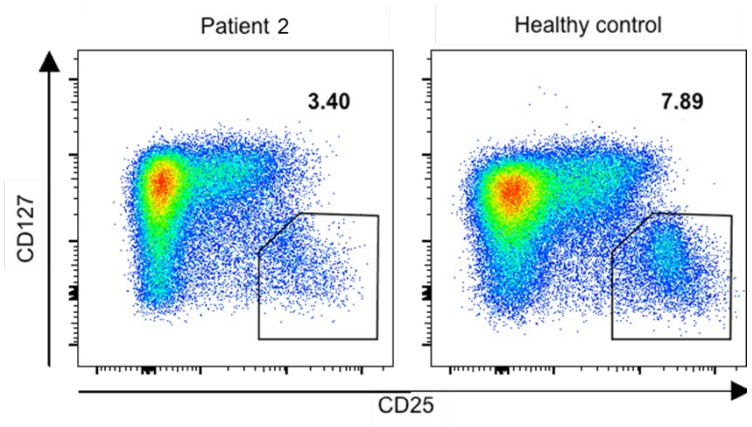

Figure 16. **Reduced T_{reg} frequency in the STAT5B-deficient Patient 2**. Gated on $CD4^+$ T cells. Histograms representative of 2 independent experiments. With permission from Elsevier Science and Technology Journals.

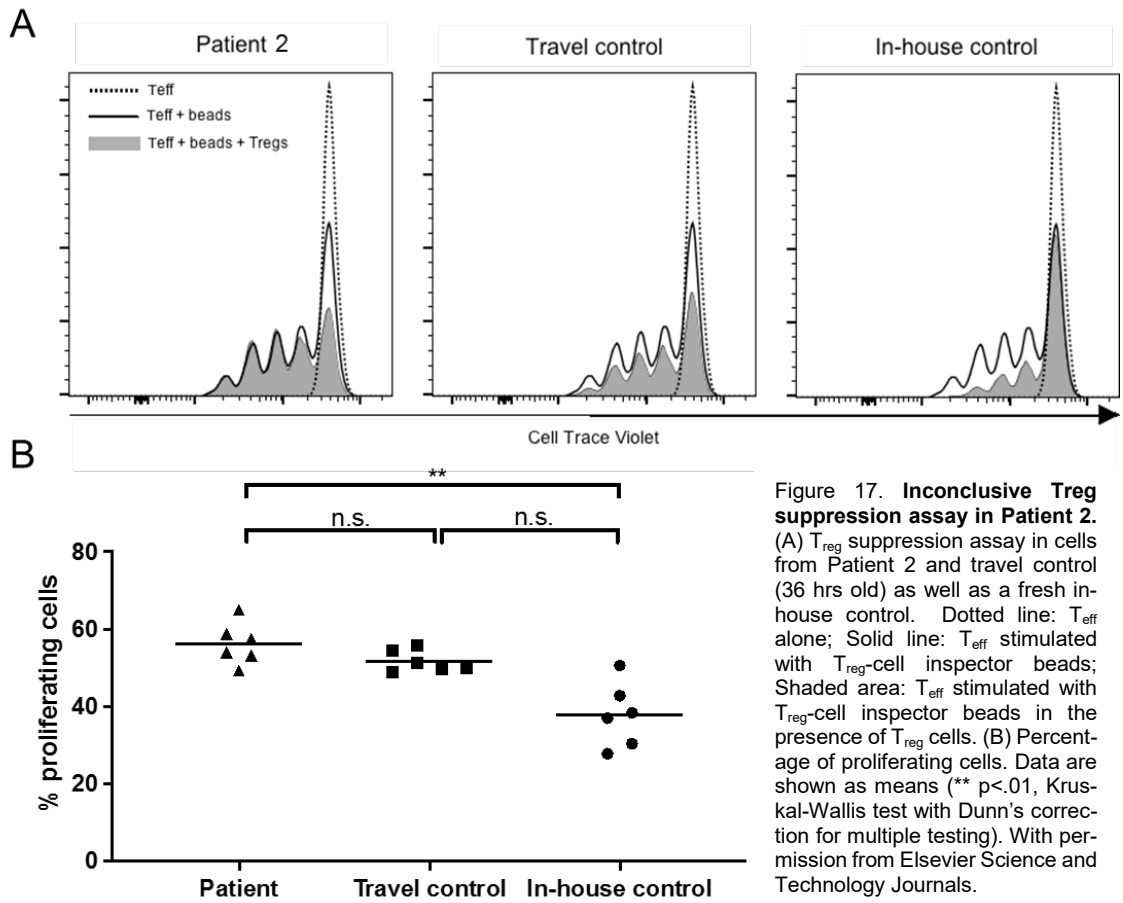

Figure 17. **Inconclusive Treg suppression assay in Patient 2.** (A) T_{reg} suppression assay in cells from Patient 2 and travel control (36 hrs old) as well as a fresh in-house control. Dotted line: T_{eff} alone; Solid line: T_{eff} stimulated with T_{reg}-cell inspector beads; Shaded area: T_{eff} stimulated with T_{reg}-cell inspector beads in the presence of T_{reg} cells. (B) Percentage of proliferating cells. Data are shown as means (** p<.01, Kruskal-Wallis test with Dunn's correction for multiple testing). With permission from Elsevier Science and Technology Journals.

3.2.3 Assessment of the Treg compartment

My part was to evaluate the T_{reg} compartment of Patient 2 in terms of size and function. Using flow cytometry, I found T_{reg} numbers decreased by approximately 50% in the patient (Fig. 16). Whereas T_{reg} function in mice is studied using adoptive transfer experiments, human T_{reg}s are usually analysed in suppression assays[156]. Here, the capacity of a certain number of T_{reg}s to limit the proliferation of autologous naïve T cells is quantified[157]. In order to control the number of Tregs in a given $CD4^+$ T cell population, cells need to be sorted by Flow cytometry. The naïve T cells, on the other hand, are best isolated using magnetic cell separation since FACS sorting negatively impacts their proliferative potential. This complex assay setup was further complicated by varying, prolonged material transfer times from Bulgaria to the UK, ranging from 36 to 72 hours. Unfortunately, we were not able to detect a significant proliferative difference between Patient 2 and healthy control cells since cells did not proliferate well enough after these transit times (Fig. 17).

3.3 JAKinib therapy in somatic STAT5B GOF disease

3.3.1 Identification of somatic *STAT5B* GOF variants in pediatric patients

Activating variants in *STAT5B* have been discovered in different haematologic malignancies. Rajala and co-workers first identified the missense variants p.Y665F and p.N642H in samples of patients suffering from large granular lymphocytic leukemia, which is characterized by a clonal expansion of cytotoxic T or NK cells. Both variants showed increased STAT5 transcriptional activity when studied in an overexpression system[158]. Subsequently, the p.N642H variant was also found amongst others in patient samples diagnosed to have T cell acute lymphoblastic leukemia or hepatosplenic T cell lymphoma and was associated with poor prognosis[159], [160].

In 2017, two patients suffering from a novel syndrome consisting of nonclonal eosinophilia, atopic dermatitis, urticarial rash and diarrhea were published[161]. In both patients the heterozygous *STAT5B* variant p.N642H was identified by WES of PBMCs. Interestingly, the variant was not present in any parental samples suggesting *de-novo* occurrence. Further investigation was carried out in one of the patients by sequencing a different tissue. Here, no variant in *STAT5B* was found indicating a somatic rather than germline origin of the sequence alteration. To test whether PBMC populations might be differentially affected by the variant, different cell populations were FACS sorted and analysed separately. It became clear, that the p.N642H variant was present in nearly half of the T cell and eosinophil compartment whereas B cell and $CD11c^+$ myeloid cells were less affected[161]. Since both lymphoid and myeloid lineages were affected, the somatic mutation was attributed to an early haematopoietic progenitor stage like the multipotent progenitor or the lymphoid-primed multipotential progenitor stage[162].

When studying $CD4^+$ T cell differentiation in both patients, a shift towards type 2 cells (T_H2) with increased IL-13 production was noted[161]. The high mutation frequency with the eosinophil compartment might be explained by enhanced responses to IL-5, another T_H2 cytokine preferentially utilizing STAT5B downstream signals and essential in the differentiation and survival of eosinophils[163], [164]. In mouse models of enhanced STAT5b activity, increased IL-3 responsiveness and thymic stromal lymphopoietin production lead to severe mast cell–driven skin inflammation resembling the atopic dermatitis phenotype seen in the two patients[165].

3.3.2 Analysing the cellular response to JAKinib therapy

Since the pathogenesis of the observed severe allergic phenotype was believed to result from enhanced T_H2 cytokine responsiveness, using JAKinibs to tune down receptor signalling upstream of STAT5 seemed an attractive, targeted treatment option. Indeed, both patients under investigation, one from the US and the other one from Ireland, responded well to ruxolitinib treatment with reduction of eosinophil counts and improvement of gastrointestinal symptoms[166]. We therefore set out to investigate whether the clinical response to JAK-inhibition was associated with an altered cellular behaviour. I was studying primary cells from the Irish patient Patient 3), analysing STAT5-phosphorylation in various leucocyte subsets stimulated with different cytokines when the patient was on or off ruxolitinib treatment (Fig. 18). Whereas most cytokine responses were not affected by the ruxolitinib treatment of Patient 3, IL-21 induced STAT5-phosphorylation in T and NK cells was found reduced.

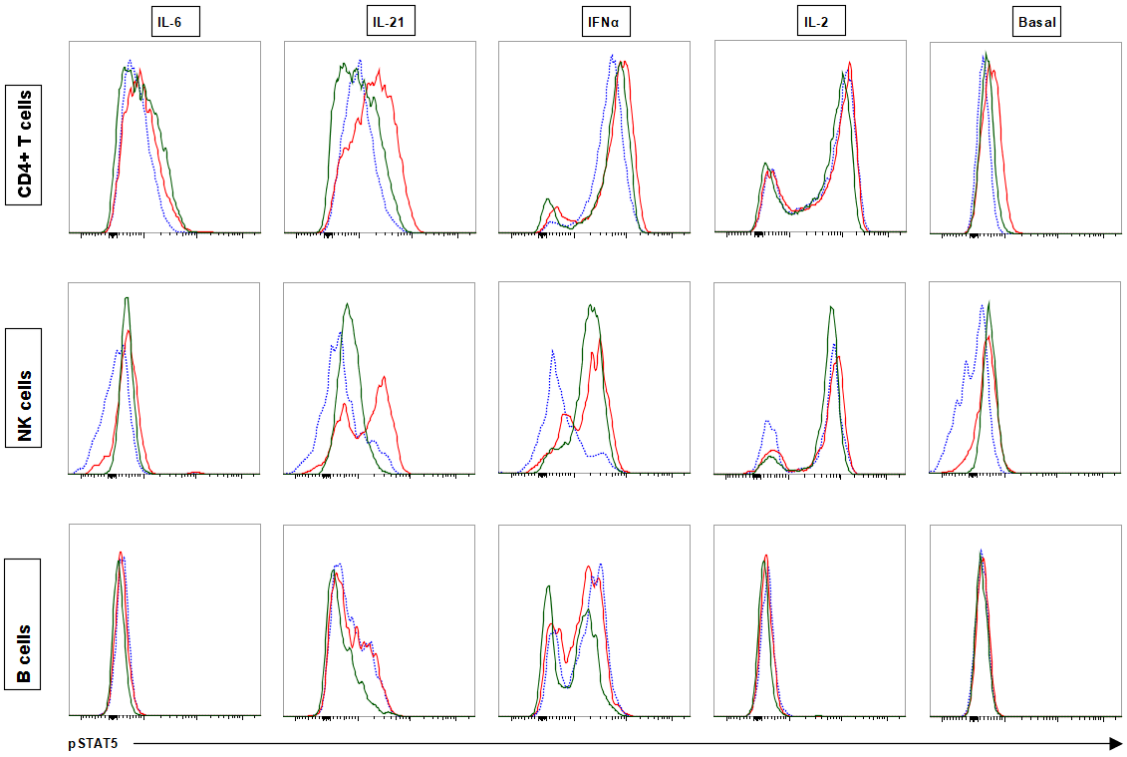

Figure 18. **Hyperphosphorylation of STAT5 following IL-21 stimulation in Patient 3 alleviated by Ruxolitinib.** STAT5 phosphorylation in basal and stimulated cells of Patient 3 compared with a healthy control (dotted blue line). The green line displays patient cell responses during Ruxolitinib treatment. The red line indicates the sample taken when the patient was off Ruxolitinib treatment. With permission from Elsevier Science and Technology Journals.

Next, we asked whether the JAKinib treatment might alter the frequency of p.N642H-containing cells potentially exerting a preferential effect on the mutated cells. Hence, I developed a FACS sorting panel allowing the simultaneous collection of T, B, and NK cell, as well as eosinophil and dendritic cell populations. Allowing DNA extraction, cells were shipped to the NIH and Chi Ma analyzed mutated allele frequencies using droplet digital PCR. Interestingly, allele frequencies were unchanged by ruxolitinib in every subset analysed[166]. This highlights the notion, that JAKinibs offer a symptomatic rather than a disease-modifying treatment to STAT5B GOF disease. In order to study the p.N642H mechanistically and in greater detail, I established an EBV-LCL from primary patient cells. Since only 20% of the patient's B cells carried the mutant allele, I single cell-sorted them to grow individual clones from single cells. Unfortunately, I failed to expand these clones sufficiently to conduct further experiments dissecting the molecular basis of the GOF behaviour.

3.4 IFNAR1 deficiency presenting with HLH

3.4.1 Case report and identification of a rare variant in IFNAR1

We studied a 15-month-old boy of consanguineous parentage (Patient 4), who presented with fever five days after the first dose of MMR vaccination to his local hospital in Slowakia. On admission, generalised lymphadenopathy, hepatosplenomegaly, and oedema were noted. Laboratory testing supported the diagnosis of HLH while no infectious agent could be identified in blood and cerebrospinal fluid (CSF). Immunosuppressive treatment for presumed HLH led to clinical improvement and the initial resolution of fever. However, fever returned a few days later while the patient was still on triple immunosuppression and the clinical situation culminated in progressive systemic inflammatory response syndrome with respiratory failure. At this point, genetic testing by whole exome sequencing was undertaken, identifying a private, homozygous missense variant c.922C>T in *IFNAR1,* leading to the introduction of a premature stop codon at position 308 (p.Q308X). Following a long recovery period, the boy was discharged on anti-infective prophylaxis while immunosuppression was discontinued. Shortly after he had to be readmitted due to fever, generalized seizures, hyponatraemia and treatment-refractory hypertension. Again, screening for viral disease in blood or CSF revealed no causative pathogen but repeated cranial magnetic resonance imaging (MRI) showed progressive cerebral atrophy and a lesion in the left hippocampus suggestive of inflammatory or infective aetiology. Patient 4 finally died of sudden cardiorespiratory failure at the age of 21 months and the family vetoed a post-mortem examination.

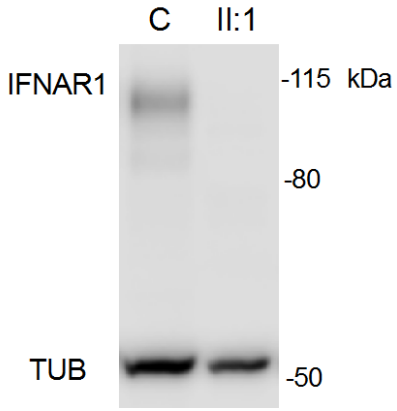

Figure 19. **Absent IFNAR1 expression in Patient 4 (P4) compared to a healthy control (HC).** Representative immunoblot from n=3 repeat experiments in fibroblasts. *TUB: α-Tubulin.* With permission from Oxford University Press-Journals.

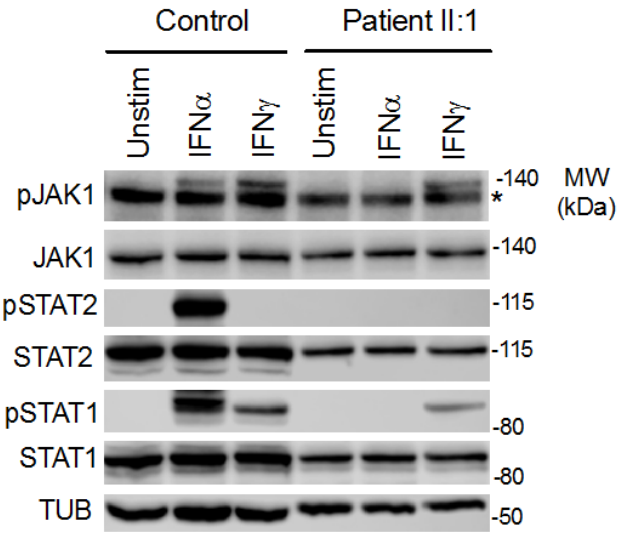

Figure 20. **Defective type I IFN signalling in Patient 4**. Signalling induction in response to 15 minutes of IFNα or IFNγ stimulation in fibroblasts of Patient 4 and a healthy control. n=3 repeat experiments. *TUB: α-Tubulin;* With permission from Oxford University Press-Journals.

3.4.2 Functional validation of the IFNAR1 variant

Since the variant introduced a premature stop codon in the third extracellular domain of the IFNAR1 protein, we hypothesised that protein expression would be lost. Indeed, I was unable to detect IFNAR1 protein using a N-terminal antibody (Fig. 19). In line with the absence of a functional type I IFN receptor on the cell surface, I failed to detect JAK1, STAT1, or STAT2 phosphorylation in response to IFNα whereas responses to IFNγ were found unaffected indicating an intact

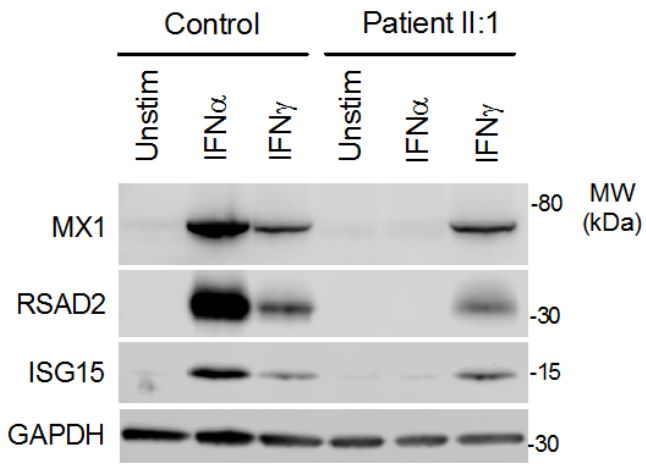

Figure 21. **Failure of ISG-induction in Patient 4.** Upregulation of different ISGs following 16 hrs of IFNα or IFN*γ* stimulation in fibroblasts of Patient 4 and a healthy control. Repeat experiments n=3. With permission from Oxford University Press-Journals.

cephalomyocarditis virus. Whereas pre-treatment with IFNα reduced virally induced cell death in control cells, this was not the case in IFNAR1-deficient cells. To finally prove the causal relationship between loss of IFNAR1 expression and susceptibility to viral disease, I complemented primary fibroblasts from Patient 4 with wild-type *IFNAR1* via lentiviral transduction. Rescue of IFNAR1 expression restored ISG production following IFNα stimulation (Fig. 22) and promoted cell survival in IFNα-pre-treated, *Encephalomyokarditis virus* (EMCV)-infected patient fibroblasts.

signalling cascade downstream of IFNGR (Fig. 20). Furthermore, I tested the upregulation of several well-described ISGs and confirmed the lack of a type I IFN response in Patient 4 (Fig. 21). My fellow PhD-student Catherine Hatton tested the susceptibility of primary fibroblasts from Patient 4 to viral infection using either Zika virus or en-

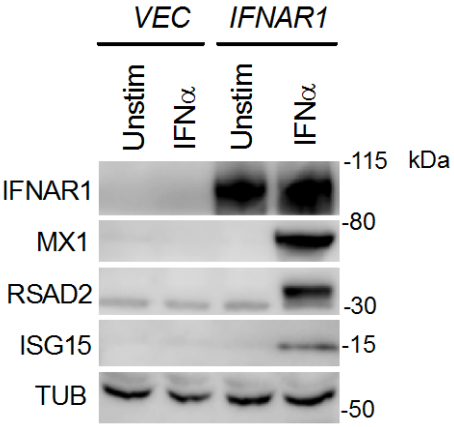

Figure 22. **Restored ISG-induction after complementation with wild-type IFNAR1.** ISG expression following IFNα stimulation for 16 hrs in fibroblasts of Patient 4 complemented with either empty vector (*VEC*) or wild-type *IFNAR1*. Representative immunoblot from n=3 independent experiments. *TUB: α-Tubulin;* With permission from Oxford University Press-Journals.

3.5 STAT2 GOF disease as a new type I interferonopathy

3.5.1 Aicardi-Goutières syndrome in two brothers

The index patient (Patient 5), born to consanguineous parents of Pakistani descent, came to medical attention at six months of age when developmental delay became apparent. Two months later, he developed a first episode of systemic inflammation trig gered by intercurrent infection with prolonged fever, hepatosplenomegaly, thrombocytopenia, raised ferritin, and elevated liver enzymes. Throughout the following year, recurrent HLH-like episodes occurred, which were accompanied by signs of neuroinflammation as evidenced by progressive intracranial calcifications, white matter changes and intracranial bleeding at the age of 1.5 years. This clinical phenotype raised the suspicion of a type I interferonopathy. As a first indication, ISG scores were repeatedly measured in whole blood and revealed substantially elevated levels comparable to known type I interferonopathies[125]. The disease was found partially responsive to dexamethasone and stabilized with the addition of ruxolitinib. However, since the diagnostic criteria for HLH were met during episodes of inflammation, the treating physicians decided to go for a replacement of the entire immune system via HSCT. During the HSCT procedure, however, the boy succumbed to overwhelming Gram-negative bacterial sepsis.

The younger brother of the index patient already displayed abnormal neurodevelopment and neuroimaging during the first months of life. He experienced apneic episodes while MRI revealed parenchymal calcifications and hemorrhage, abnormal cerebral white matter, and brainstem and cerebellar atrophy. His ISG scores analysed in whole blood were also elevated alongside anaemia and increased ferritin levels. After ruxolitinib had been commenced, ISGs levels returned to normal and an initial improvement of the respiratory situation was seen. Given the irretrievable neurological damage, however, the palliative care team was involved and the patient died at the age of 3 months.

3.5.2 Identification of a disease-causing homozygous *STAT2* variant

Whole exome sequencing of the family revealed the homozygous missense variant c.442C>T in the *STAT2* gene, predicted to result in the amino acid change p.R148W and segregating with disease in the given kindred. Christopher Duncan and other members of the Hambleton lab demonstrated that STAT2 protein expression was not affected by the variant, but primary fibroblasts of Patient 5 displayed enhanced sensitivity to type I IFN resulting in increased expression of ISGs following IFNα stimulation in accordance with the whole blood derived ISG scores. However, there was no evidence of basal activity in the absence of IFNα stimulation. I was involved to study STAT phosphorylation in EBV-LCLs derived from Patient 5 and the unaffected mother. It became clear, that homozygous, but not heterozygous $STAT2^{R148W}$ cells show an abnormally prolonged phosphorylation of both STAT1 and STAT2 until 24 hrs of IFNα stimulation indicating unrestrained IFNAR signalling (Fig. 23). Hence, a defect in negative regulation of IFNAR signalling was hypothesised rather than an increased type I IFN production as evident in most forms of Aicardi-Goutières syndrome.

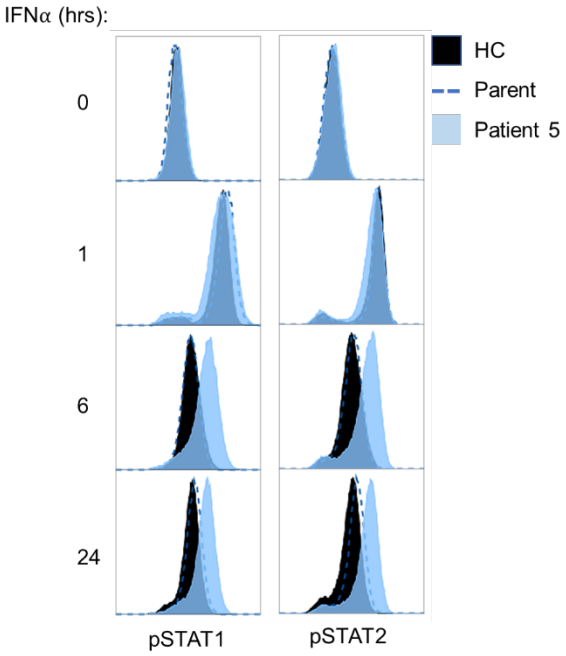

Figure 23. **Prolonged IFNAR signalling in STAT2 GOF cells**. IFNAR signalling in EBV-LCLs from Patient 5 (light blue) compared to heterozygous parental (dashed blue line) or healthy control cells (black) following stimulation with IFNα for the indicated time points. Representative flow plots of 3 independent experiments. With permission from the American Association for the Advancement of Science.

In search for the underlying mechanism, STAT2 dephosphorylation was assayed using the kinase inhibitor staurosporine and found comparable to control cells. Next, known negative regulators of IFN signalling were investigated. Normal SOCS1 and SOCS3 mRNA levels in whole blood samples and regular STAT1 and STAT3 phosphorylation kinetics following IFNγ and IL-6 stimulation, respectively, argued against involvement of these classical inhibitors of JAK-STAT signalling. Therefore, a potential contribution of USP18, a specific inhibitor of type I IFN signalling, to the observed cellular behaviour was studied. Using co-immunoprecipitation in an overexpression system, an impaired interaction between the $STAT2^{R148W}$ protein and USP18 became apparent. The insensitivity to USP18-mediated termination of IFNAR signalling in $STAT2^{R148W}$ cells was finally confirmed through overexpression of USP18 in cells of Patient 5 and USP18 knockdown in control cells.

In summary, studying this family elucidated homozygous *STAT2* variants as a new IEI and type I interferonopathy, revealing the dual role of STAT2 in both promoting IFNAR signalling as part of the ISGF3 TF complex and on the other hand confining type I IFN responsiveness by concerted action in conjunction with USP18.

3.6 Hyperinflammation in STAT2 and IRF9 deficiency

3.6.1 Prolonged IFNAR signalling in ISGF3 component-deficient cells

For the STAT2 GOF project, I was studying the phosphorylation kinetics of different STAT molecules within the same cell by flow cytometry. As a negative control, I had included EBV-LCLs from a patient with a novel homozygous LOF variant in *STAT2.* Surprisingly, I observed less intense, but substantially prolonged STAT1 phosphorylation in cells deficient for STAT2 (Fig. 24). The lack of pSTAT1 signal attenuation for as long as 48 hrs of stimulation with IFNα was rather unexpected and therefore thought provoking and ultimately hypothesis generating. While reduced responsive-

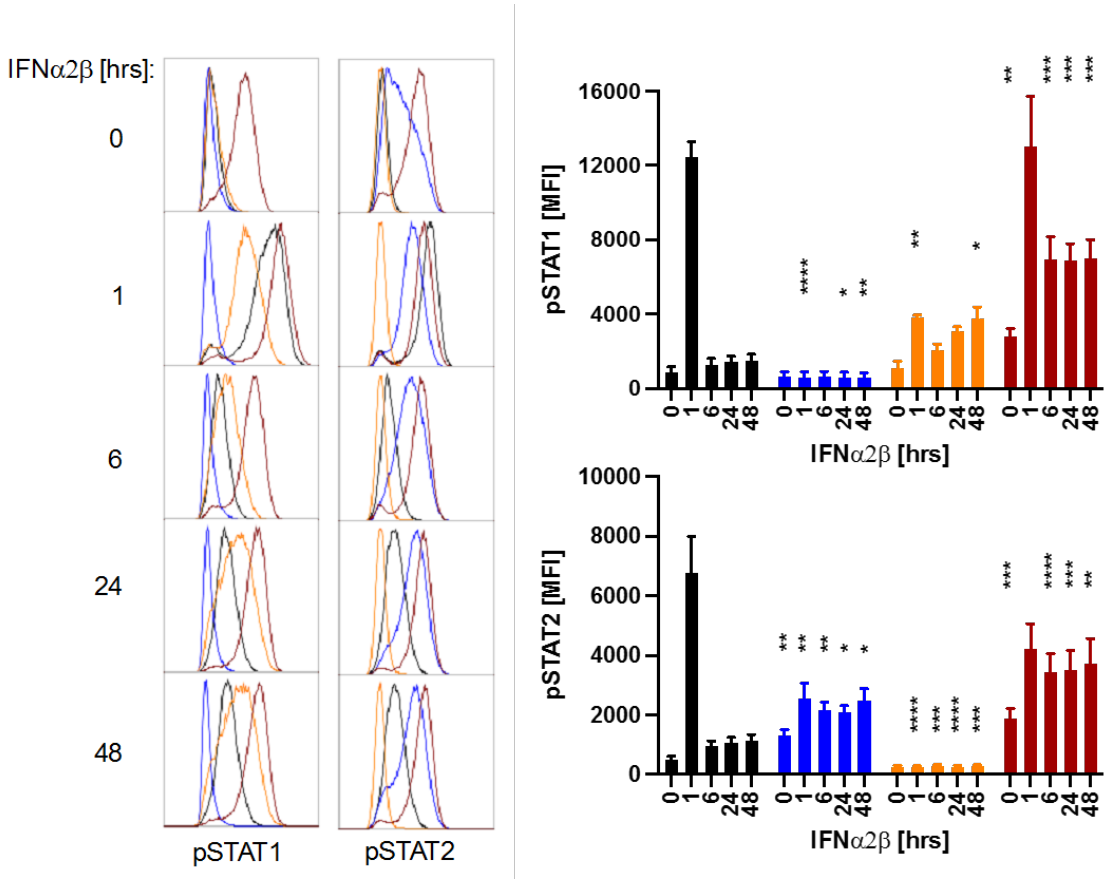

Figure 24. **Prolonged STAT-phosphorylation in ISGF3-deficient cells.** (A) IFNAR signalling in STAT1$^{-/-}$ (blue), STAT2$^{-/-}$ (orange), and IRF9$^{-/-}$ (red) EBV-LCLs compared to healthy control cells (black) stimulated with IFNα2b for the indicated time points. Representative histograms of n=4 repeat experiments are shown. Bar graphs display mean fluorescence intensity (MFI) of (B) pSTAT1 or (C) pSTAT2. Data are shown as mean ± SEM (* p<.05, ** p<.01, *** p<.001, **** p<.0001, one-way ANOVA with Tukey correction for multiple comparisons). With permission from Elsevier Science and Technology Journals.

ness of STAT1 to IFNα in the absence of STAT2 fitted with the well-studied failure to mount an antiviral response in these cells, we wondered whether there might be an additional problem to switch the insufficient response off again. We therefore decided to test in parallel patient cells deficient for the other two components of the ISGF3 complex. These cells were kindly provided

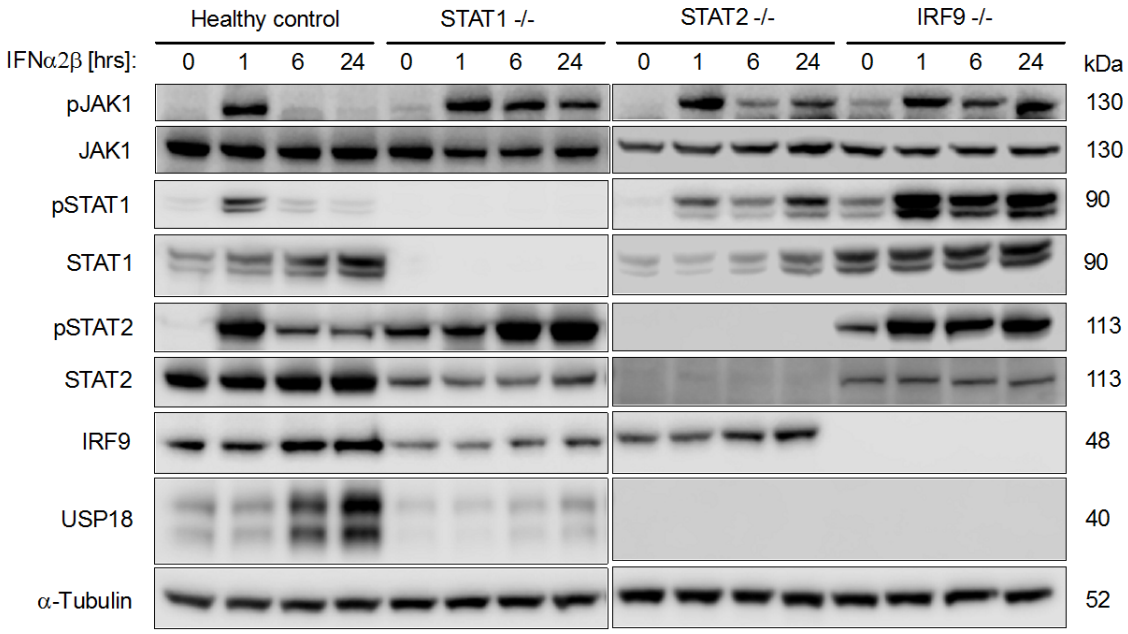

Figure 25: **Ongoing JAK1-phosphorylation in ISGF3-deficient cells.** Representative immunoblot showing signalling intermediates following IFNα2b stimulation for the indicated time points in STAT1, STAT2, and IRF9-deficient as well as healthy control EBV-LCLs. Repeat experiments n=3. With permission from Elsevier Science and Technology Journals.

by Fabian Hauck in the case of STAT1 deficiency[167], while Hugh T. Reyburn sent IRF9-deficient cells[109]. In line with our previous results, I noted prolonged phosphorylation of the residual STAT molecule following IFNα treatment. Especially in cells lacking IRF9, both pSTAT1 and pSTAT2 levels were significantly upregulated at late time points compared to control cells (Fig. 24). I confirmed and extended these results using Western Blot, where we also detected prolonged JAK1 phosphorylation upstream of the STAT molecules indicating prolonged IFNAR signalling (Fig. 25). Clinically, in both STAT2 deficiency and IRF9 deficiency, episodes of hyperinflammation have been reported in the absence of viral infection potentially indicating an immune dysregulatory component of these diseases.

3.6.2 Failure of USP18-induced negative regulation

Well aware of the essential role of USP18 in IFNAR signalling desensitization, we hypothesized a failure to upregulate USP18 to be causative of the ongoing signalling and could demonstrate this both on *USP18* transcript as well on protein level (Fig. 25). Whereas *USP18* mRNA expression peaked after 24 hrs in control cells, all ISGF3-deficient cell lines showed severely impaired *USP18* induction (Fig. 26). Although some residual transcription was detectable in STAT1$^{-/-}$ cells, possibly reflecting the ability of residual STAT2:IRF9 containing complexes to mediate the expression of certain ISRE-containing ISGs in the absence of STAT1[168], [169], no induction of *USP18* could be observed in STAT2$^{-/-}$ or IRF9$^{-/-}$ cells even as late as 48 hrs after

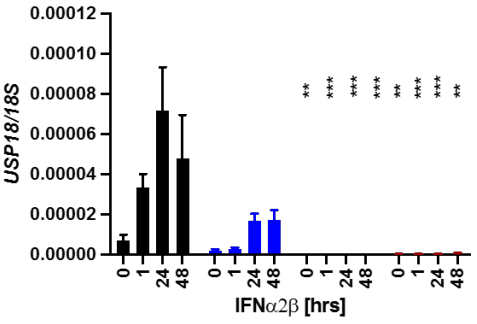

Figure 26. **Failure of USP18 induction**. Transcriptional induction of USP18 in STAT1$^{-/-}$ (blue), STAT2$^{-/-}$ (orange), and IRF9$^{-/-}$ (red) EBV-LCLs compared to healthy control cells (black) stimulated with IFNα2b for the given time points. Data are shown as mean ± SEM ** p<.01, *** p<.001, one-way ANOVA with Tukey correction for multiple comparisons). Repeat experiments n=3. With permission from Elsevier Science and Technology Journals.

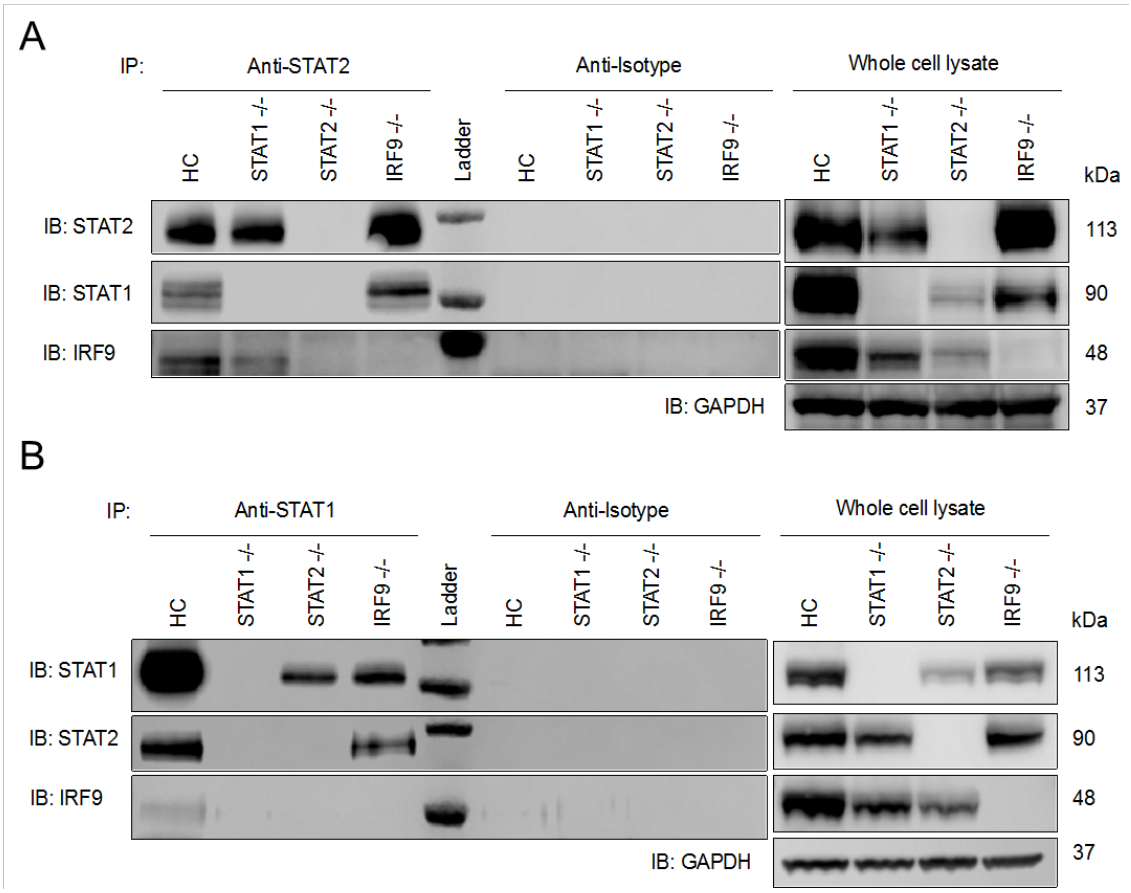

Figure 27. **Preserved dimeric interactions of ISGF3 components.** Coimmunoprecipitation of (A) STAT1 and (B) STAT2 in EBV-LCLs stimulated with IFNα2b for 15 minutes. Representative images of n=3-4 independent experiments. *IP: Immunoprecipitation; IB: Immunoblot;* With permission from Elsevier Science and Technology Journals.

onset of IFNα stimulation. The preserved interaction of STAT2 and IRF9 in the absence of STAT1 was shown by co-immunoprecipitation experiments in the primary EBV-LCLs (Fig. 27). To check for a potential contribution of other negative regulators, namely SOCS1 and SOCS1, we checked their induction by RT-qPCR. SOCS1 induction was not significantly reduced in any ISGF3 component-deficient cells, whereas SOCS3 was induced normally, displaying even enhanced tran-

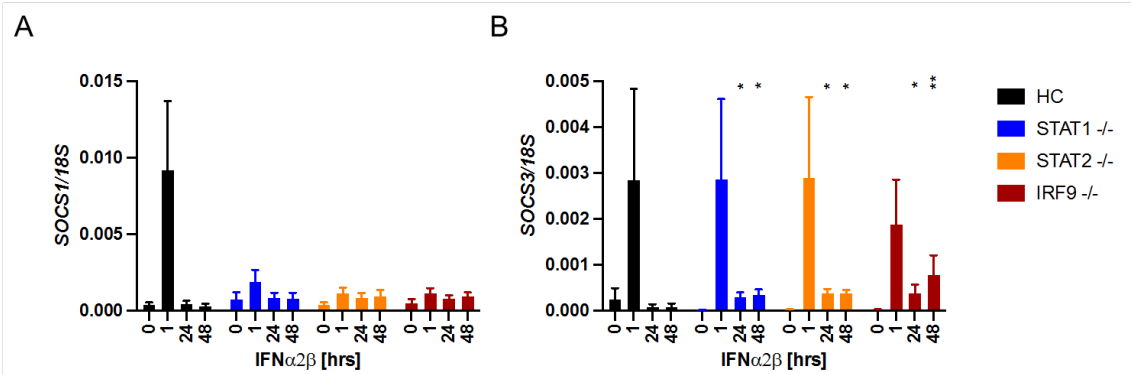

Figure 28. **Sustained negative regulation via the SOCS proteins.** Transcriptional induction of (A) *SOCS1* and (B) *SOCS3* following IFNα2b stimulation over time measured by RT-qPCR. Repeat experiments n=3-4. Data are presented as means ± SEM (* p<.05, ** p<.01, one-way ANOVA with Tukey correction for multiple comparisons). With permission from Elsevier Science and Technology Journals.

scription at later time points (Fig. 28). The observed failure of the SOCS proteins to compensate for missing USP18 action is in line with our previous data in the STAT2 GOF subjects as well as data in mice[170].

3.6.3 GAS-dominated transcriptional output in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells

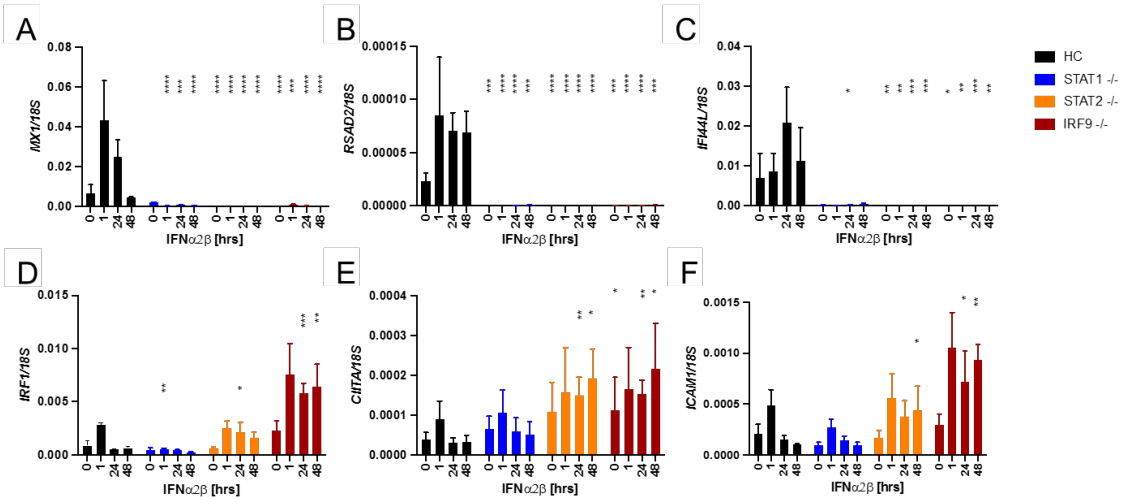

Figure 29. **Missing induction of antiviral genes and accumulation of transcripts controlled by IFNγ over time**. RT-qPCR analysis of ISGs in response to IFNα2b stimulation for the indicated times. Expression of classical antiviral genes (A) *MX1*, (B) *RSAD2*, (C) *IFI44L*. Induction of known IFNγ-activated genes (D) *IRF1* (E) *CIITA*, (F) *ICAM1*. Repeat experiments n=3-4. Data are presented as means ± SEM (* p<.05, ** p<.01, *** p<.001, **** p<.0001, one-way ANOVA with Tukey correction for multiple comparisons). With permission from Elsevier Science and Technology Journals.

To assess the transcriptional consequences of such prolonged IFNAR signalling, we examined the upregulation of some well-described ISGs important to induce an antiviral state in response to IFNα stimulation. We found no induction of either *MX1*, *RSAD2* or *IFI44L* transcription following IFNα treatment in cell lacking a full ISGF3 complex (Fig. 29), a finding that corresponds well to the clinically observed viral susceptibility of the patients. Interestingly, we detected an increased transcription of *IRF1* in both STAT2$^{-/-}$ and IRF9$^{-/-}$ cells after 24 hrs of IFNα stimulation. *IRF1* is a classical target gene of STAT1-homodimers constituting the GAF, as already mentioned. In-

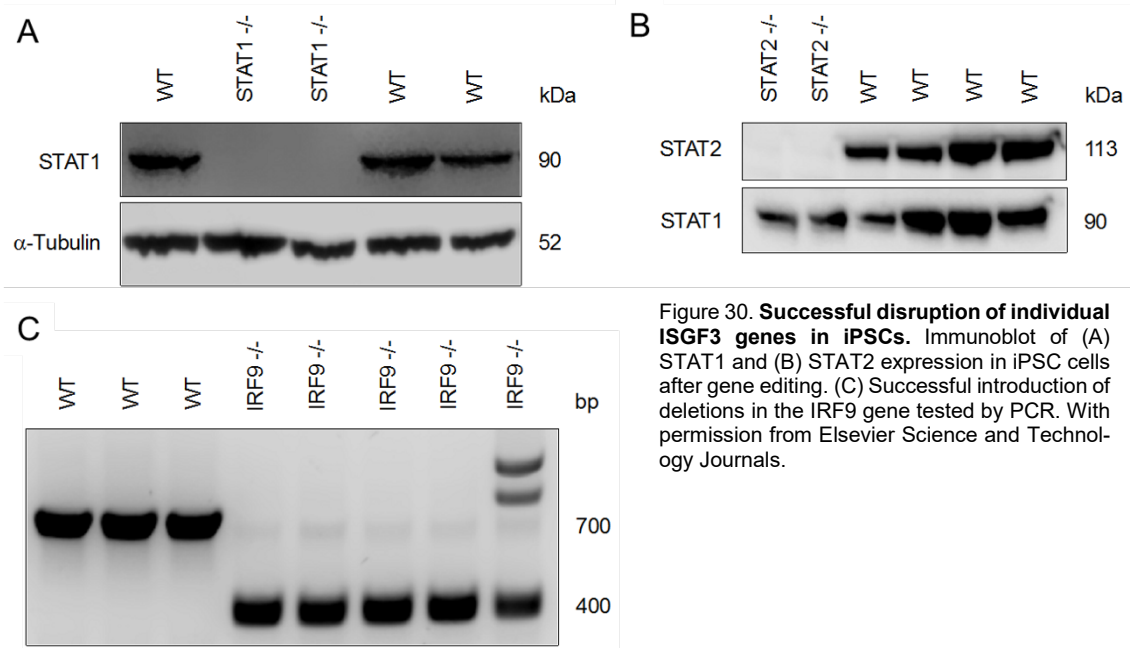

Figure 30. **Successful disruption of individual ISGF3 genes in iPSCs.** Immunoblot of (A) STAT1 and (B) STAT2 expression in iPSC cells after gene editing. (C) Successful introduction of deletions in the IRF9 gene tested by PCR. With permission from Elsevier Science and Technology Journals.

creased *IRF1* transcription had already been noted in STAT2-deficient cells after 10 hrs of IFNα treatment[171]. In both STAT2- and IRF9-deficient cells, however, the formation of GAF complexes is expected to remain intact, and I therefore went on to check other genes known to harbour GAS sites. I detected heightened transcription of classical IFNγ-induced genes like *CIITA*, the master regulator of MHC class II expression in antigen-presenting cells and *ICAM1*, encoding

a protein expressed on macrophages and lymphocytes that facilitates cell-cell interaction including immune synapse formation. Looking at these data, we refined our hypothesis and asked whether a GAF-driven transcriptional response in STAT2- and IRF9-deficient patients might underlie the observed inflammatory manifestations[172].

3.6.4 Similar signalling kinetics in STAT2$^{-/-}$ and IRF9$^{-/-}$ macrophages

To test this hypothesis, a model system of greater biological relevance to inflammation seemed necessary since EBV-LCLs are themselves virally infected cells and thus not ideal to study antiviral responses. Macrophages, known as the key cell type driving HLH pathology, appeared an attractive cell type to study. We therefore decided to employ macrophages differentiated from human iPSC, which had been gene edited using CRISPR/Cas9 technology. Instead of taking primary patient cells as a source of iPSCs thereby preserving the individual genetic background as well as the specific mutations of the patients, we created iPSC lines with disruptions of the *STAT1*, *STAT2* or *IRF9* gene, respectively, on an isogenic background to optimize the model system for mechanistic studies by controlling the aforementioned individual factors. STAT1-deficient iPSCs were generated as a negative control for GAF-driven inflammation. To confirm the successful introduction of frameshift mutations in the target genes preventing protein expression, I tested the expression of STAT1 and STAT2 in iPSC (Fig. 30A-B). Since I found IRF9 not to be expressed in iPSCs, the presence of the desired deletion was checked using PCR (Fig. 30C).

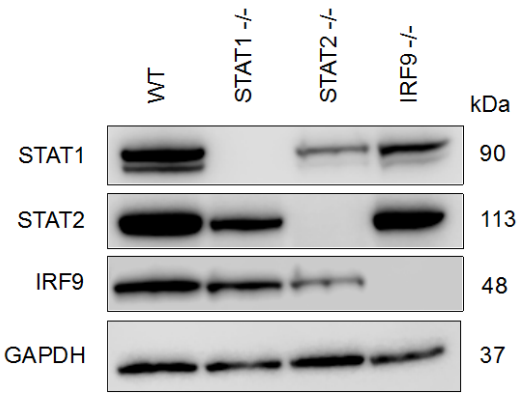

Figure 31. **Absent expression of ISGF3 components in iPSC-derived macrophages.** Immunoblot testing expression of ISGF3 components in gene-edited iPSC-derived macrophages. Repeat experiments n=3. With permission from Elsevier Science and Technology Journals.

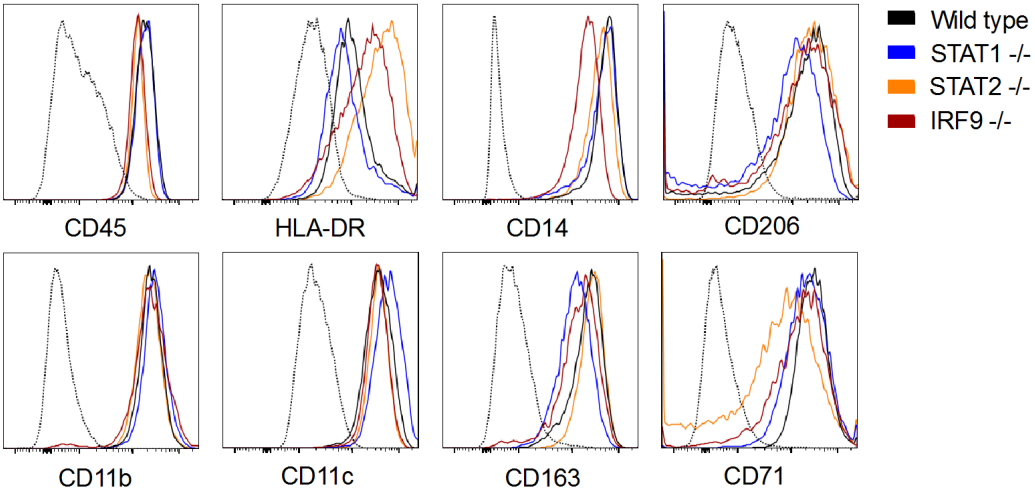

Figure 32. **Macrophage-like phenotype of iPSC-derived macrophages.** Representative flow cytometry histograms of iPSC-macrophage surface marker expression. The dotted lines indicate isotype controls. Repeat experiments n=2. With permission from Elsevier Science and Technology Journals.

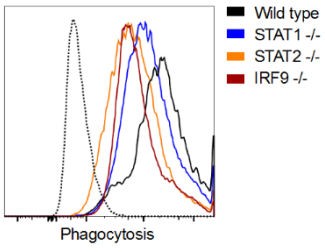

Figure 33. **Comparable phagocytic activity of iPSC-macrophages.** Fluorescent bead uptake at 37°C versus 4°C (dotted line, negative control). Repeat experiments n=2. With permission from Elsevier Science and Technology Journals.

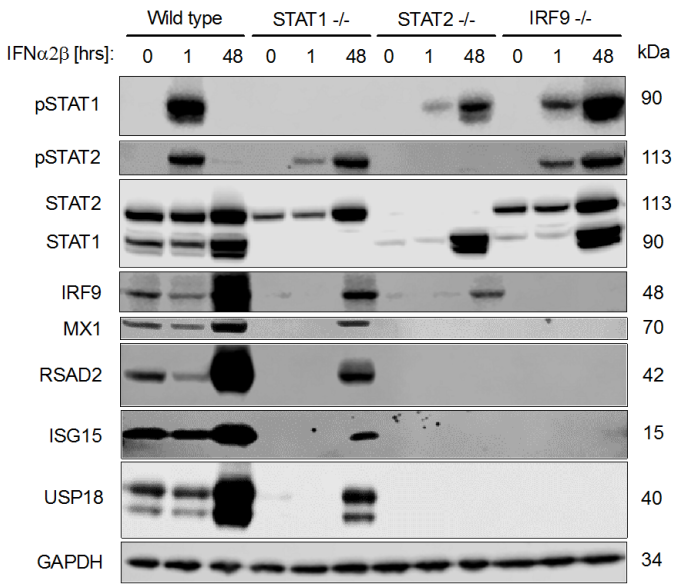

Figure 34. **Prolonged STAT-phosphorylation and altered ISG expression in ISGF3-deficient iPSC-macrophages.** Signalling kinetics and expression of ISGs in iPSC-macrophages stimulated with IFNα2b for up to 48 hrs. Repeat experiments n=3. With permission from Elsevier Science and Technology Journals.

Having successfully created different clones of iPSCs harbouring loss-of-expression mutations in either *STAT1*, *STAT2* or *IRF9*, I differentiated the iPSC into macrophage-like cells using a published protocol, which has the advantage of using feeder- and serum-free culture conditions and produces cells over a period of 12 weeks on average[123]. Another reason to use cell lines derived from gene edited iPSCs was the fact that macrophages are notoriously hard to transfect or transduce since they are experts in recognizing foreign nucleic acids[123]. The loss of the respective protein expression was confirmed by immunoblot (Fig. 31). To check whether the differentiated cells resemble a macrophage-like phenotype, I checked the surface expression of multiple macrophage markers by flow cytometry (Fig. 32). Additionally, I confirmed their phagocytic capability using pH-sensitive fluorescent beads (Fig. 33).

Next, I explored whether IFNAR signalling kinetics were altered in the macrophage-like cells in a manner analogous to patient EBV-LCL (Fig. 34). I observed a similarly prolonged phosphorylation of residual STAT molecules up to 48 hrs of stimulation with IFNα, a complete failure to upregulate USP18 in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells, as well as a defect in upregulating antiviral proteins like MX1, RSAD2 or ISG15. Again, some induction of both USP18 and antiviral protein was seen in STAT1$^{-/-}$, corresponding to the data in EBV-LCLs lacking STAT1. I did not confirm prolonged JAK1 phosphorylation in macrophage-like cells since both the JAK1 as well as the pJAK1 antibodies require high protein concentrations and cell numbers were limited.

3.6.5 Transcriptional changes in STAT2$^{-/-}$ and IRF9$^{-/-}$ macrophages reveal an IFNγ-like pattern and altered time-course

Next, we sought to profile the transcriptional response of these cells in a comprehensive manner. Since the amount of RNA extracted from the macrophage-like cell lines was too low for standard RNA sequencing, we utilized the Nanostring Host Response Panel including 785 genes relevant to immune functions. We analysed differentially expressed genes in two pooled wild-type lines and compared to the lines KO for either STAT1, STAT2 or IRF9. When stimulating the cells for 1 hr with IFNα, all three KO lines displayed a marked failure to up-regulate ISGs when compared to WT cells, with STAT2$^{-/-}$ and IRF9$^{-/-}$ cells showing a strikingly similar pattern (Fig. 35). Conversely, the response to IFNγ was preserved in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells, with STAT1$^{-/-}$ iPS-macrophages serving as an ideal negative control in this experimental setting (Fig. 36).

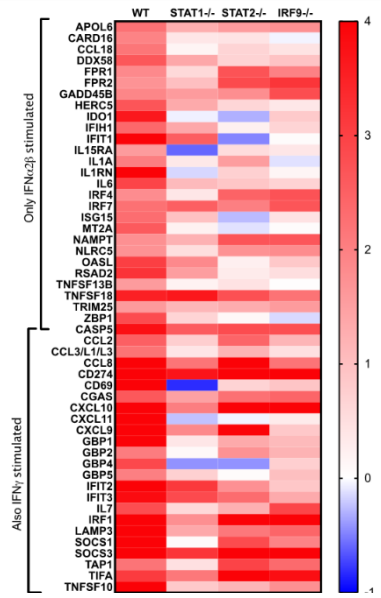

Figure 35. **Reduced IFNα2b-induced transcription of ISGs in iPSC-macrophages.** Transcriptional response in iPS-macrophages of the indicated genotype following 1 hr of IFNα2b stimulation. Only the genes with >1.5 fold induction in both WT lines are displayed. With permission from Elsevier Science and Technology Journals.

The failure of negative regulation, previously observed in the EBV-LCLs, became evident when I compared the transcriptional response to IFNα at 1 and 48 hrs of stimulation displaying only the genes who had been induced more than 1.5-fold in WT cells after 1 hr of stimulation (Fig. 37). Whereas in WT cells, most of the initially upregulated genes show decreasing expression after 48 hrs, STAT2$^{-/-}$ and IRF9$^{-/-}$ cells again showed a distinct pattern with a larger transcriptional response at the later time point. However, these delayed transcriptional changes were also qualitatively different and principal component analysis (PCA) revealing the induction of an IFNγ-like transcriptional profile in STAT2- and IRF9-deficient cells, whereas the STAT1$^{-/-}$ clustered together with the WT cells (Fig. 38). This is in line with the preserved, albeit reduced capacity of STAT1$^{-/-}$ cells to induce proteins like MX1, RSAD2 and ISG15 in response to IFNα. With the help of Helen Griffin, I was able to look at the promoter regions i.e., within 1000 bp of their

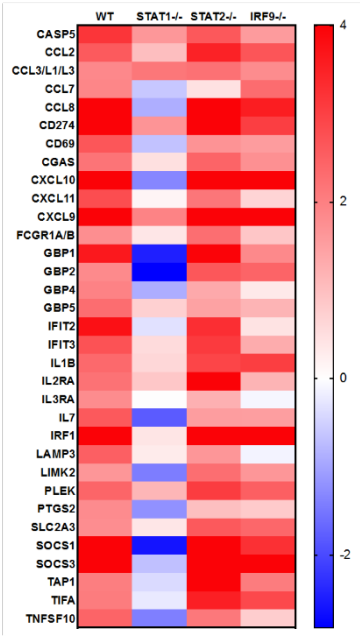

Figure 36. **Preserved transcriptional response to IFNγ in STAT2- and IRF9-deficient iPSC-macrophages.** ISG induction following IFNγ stimulation for 1hr in iPSC-macrophages of the indicated genotype. Only the genes with >1.5 fold induction in both WT line are displayed. With permission from Elsevier Science and Technology Journals.

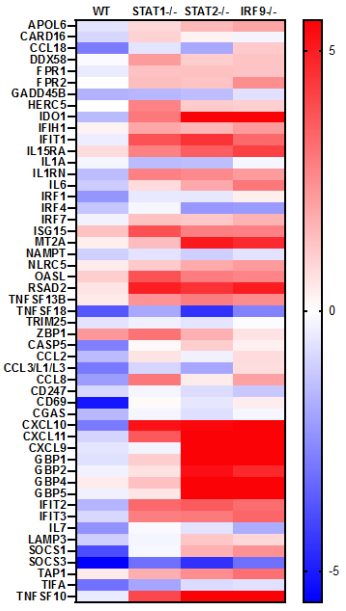

Figure 37. **Failure of negative transcriptional regulation in ISGF3-deficient iPSC-macrophages.** Transcriptional changes comparing 1 hr and 48 hrs of IFNα2b stimulation in iPSC-macrophages. Only the genes displayed in Fig. 35 are shown. With permission from Elsevier Science and Technology Journals.

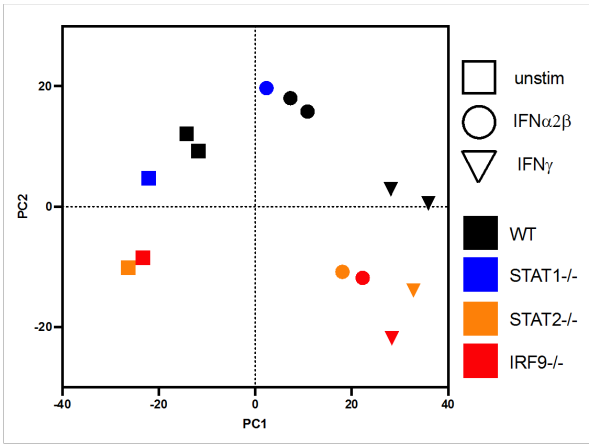

Figure 38. **Similar transcriptional profile in STAT2- and IRF9-deficient iPSC-macrophages following long-term IFNα2b or IFNγ stimulation.** Principal-component analysis (PCA) of the transcriptional output in iPSC-macrophages after 48 hrs of either IFNα2b or IFNγ stimulation. PCA1 accounts for 46% of the variability, PC2 for 19%. Square: unstimulated; circle: IFNα2b; triangle: IFNγ. With permission from Elsevier Science and Technology Journals.

respective promoter sequences, of genes differentially expressed in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells compared to WT following 48 hrs of IFNα stimulation. This analysis revealed a trend towards enrichment of GAF or IRF1-binding motifs in STAT2- and IRF9-deficient compared to WT cells (Fig. 39). In accordance, among the pathways upregulated in STAT2$^{-/-}$ and IRF9$^{-/-}$ iPSC-derived macrophages after 48 hrs of IFNα stimulation compared to WT were IFNγ signalling, antigen processing and presentation, the immune response to tuberculosis and proteasomal degradation, more typically associated with type II than type I IFN responses. We thus confirmed a transcriptional shift towards an IFNγ-like pattern following IFNα stimulation.

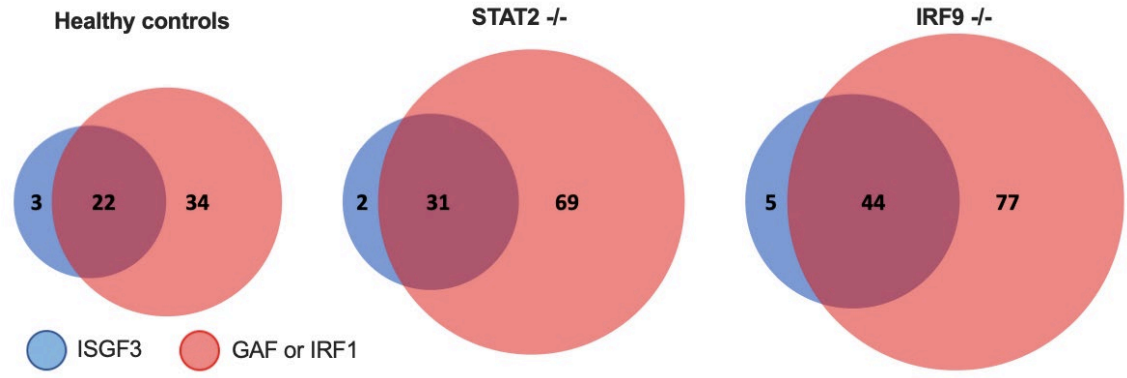

Figure 39. **Increased appropriation of IFNγ-controlled promoter sequences in STAT2- and IRF9-deficient iPSC-macrophages.** Numbers of differentially expressed genes with predicted binding sites for the indicated transcription factors ISGF3 or GAF and IRF1 within 1,000 bp of their promoter sequence for WT, STAT2$^{-/-}$ and IRF9$^{-/-}$ iPSC-macrophages following 48 hrs of IFNα2b stimulation. With permission from Elsevier Science and Technology Journals.

3.6.6 Increased activation and TNFα production in STAT2$^{-/-}$ and IRF9$^{-/-}$ macrophages

To test whether this transcriptional shift translates to the protein level and to consider the wider impact on macrophage activation, I studied the upregulation of macrophage activation markersfollowing IFNα stimulation using flow cytometry (Fig. 40). Whereas in WT cells, exposure to IFNα did not alter the expression levels of HLA-DR, ICAM1 or IRF1, IFNγ stimulation induced increasing protein expression when stimulated for up to 72 hrs. In STAT2$^{-/-}$ and IRF9$^{-/-}$ cells, however, IFNα stimulation led to significantly increased expression of all three markers, comparable to that seen with IFNγ stimulation of WT or mutant cells. As expected, STAT1$^{-/-}$ cells, used as a negative control, did not show substantial changes with either IFNα or IFNγ stimulation.

Finally, we aimed to study the production of inflammatory cytokines in IFNα or IFNγ treated iPSC-derived macrophages. Since stimulation with IFN alone is not sufficient to induce cytokine production, we primed cells for 48 hrs with either type I or type II IFN and used lipopolysaccharide (LPS) as a second stimulus (Fig. 41). IFNγ has been reported to prime macrophages for enhanced responsiveness to Toll-like receptor (TLR) ligands and to thus augment the TLR-induced expression of inflammatory cytokines[173]. Pre-treating the cells with IFNγ resulted in a comparable TNFα across all cell lines except for STAT1-deficient cells as expected. IFNα, however, only led to a modest increase in WT and interestingly also STAT1$^{-/-}$ cells, whereas in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells, TNFα production in response to IFNα even exceeded the synthesis induced by IFNγ. Thus we confirmed the presence

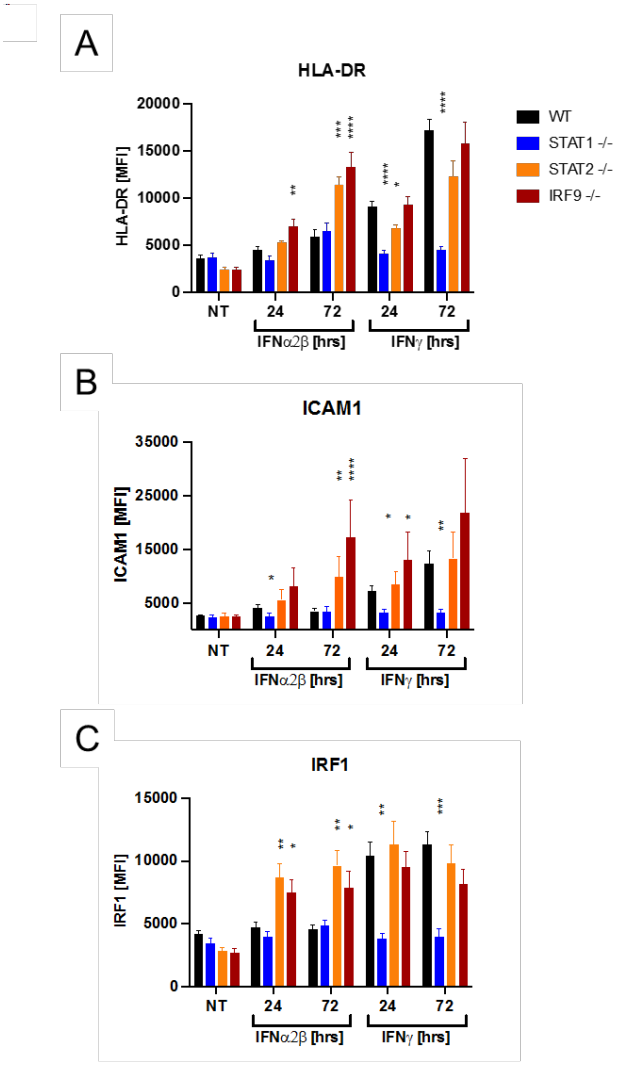

Figure 40. **Heightened expression of activation markers in iPSC-macrophages lacking STAT2 or IRF9.** Macrophage activation in response to IFN stimulation. Upregulation of surface markers (A) HLA-DR, (B) ICAM1 and the intracellular transcription factor (C) IRF1 in iPSC-macrophages following stimulation with IFNα2b or IFNγ. Repeat experiments n=3. Data are presented as mean ± SEM (* p<.05, ** p<.01, *** p<.001, **** p<.0001, one-way ANOVA with Tukey correction for multiple comparisons). With permission from Elsevier Science and Technology Journals.

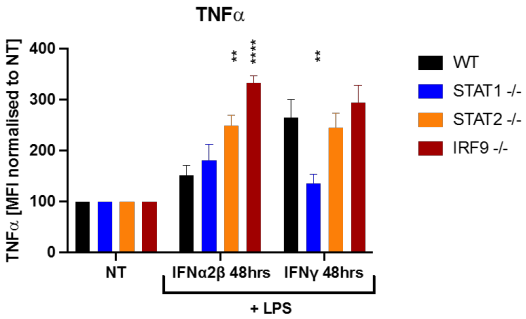

Figure 41. **Increased TNFα production in STAT2- and IRF9-deficient iPSC-macrophages.** Flow cytometric analysis of intracellular TNFα accumulation. Cells were primed with IFNα2b or IFNγ for 48 hrs before LPS was added as a second stimulus. Repeat experiments n=3. Data are presented as mean ± SEM (** p<.01, **** p<.0001, one-way ANOVA with Tukey correction for multiple comparisons). With permission from Elsevier Science and Technology Journals.

of a proinflammatory cytokine milieu following IFNα exposure in iPSC-derived macrophages deficient for STAT2 or IRF9.

4. Discussion

4.1 IL2RB deficiency as a new IEI

4.1.1 Insights into human IL-2 biology

Having discovered the homozygous *IL2RB* variant in the sequencing data of Patient 1 we searched the literature to get a first estimate of its potential impact. The striking phenotypical overlap between Patient 1 and the published KO mouse models already indicated a severely affected *IL2RB* function. The first *Il2rb* KO mouse model had been published in 1995[40]. Suzuki and colleagues reported that homozygous *Il2rb*$^{-/-}$ mice developed splenomegaly and lymphadenopathy as well as autoimmune-haemolytic anaemia at the age of three weeks[40]. Within the enlarged lymphatic tissue, activated T cells were found, which led to an increased differentiation of B cells into plasma cells. Besides massively elevated IgG and IgE levels, high concentrations of anti-nuclear and anti-DNA autoantibodies were detected. When the animals died at about 12 weeks of age from autoimmunity and progressive anaemia, a marked infiltrative granulocytopoiesis was evident in the bone marrow.

This description closely mirrors the clinical situation of Patient 1 with autoimmune cytopenias, anti-nuclear antibodies and hypergammaglobulinaemia. Only an infiltrative granulocytopoiesis was not present in Patient 1. Interestingly, the autoimmune phenotype as well as the hypergammaglobulinaemia seen in *Il2rb* KO mice could be prevented by early CD4$^+$ T cell depletion[40]. This first hint of a CD4$^+$ T cell driven immunopathology preceded the discovery of T_{reg} cells as central effectors of peripheral tolerance. However, the absence of CD25^{high} FOXP3$^+$ CD4$^+$ in *Il2rb* KO situations was confirmed later on. Surprisingly, IL-2 was found dispensable for the development of T_{reg}s in the thymus but crucial for their survival and competitive fitness in the periphery[174], [175]. The aberrant population of CD25$^-$ FOXP3^{low} CD4$^+$ T cells found in Patient 1 had also been noted in KO mice before[128]. Bayer and colleagues demonstrated that survival of these cells in the periphery is critically dependent upon IL-7 signalling, which fits with our observation of heightened IL-7 responses in primary T cells of Patient 1. Taken together, these data indicate a very similar role of IL-2 signalling with regard to T cell development and T_{reg} survival in humans and mice. Moreover, they contrast with a single case report of an infant boy, who presented with an NK cell deficient form of severe combined immunodeficiency (SCID) and in whom a severe reduction of IL2Rβ expression had been attributed as disease-causing[176]. Importantly, targeted sequencing of this patient had not revealed any mutation in the *IL2RB* gene and therefore a different aetiology of the patient's phenotype has to be assumed.

4.1.2 Shared differentiation defect of cytotoxic lymphocytes

In 1997, an additional defect of NK cell development in *Il2rb*$^{-/-}$ mice was reported[129]. Besides a reduced number of peripheral NK cells, their capacity to kill target cells as well as to produce IFNγ were nearly absent. This phenotype is clearly different from what we observed in NK cells from Patient 1: We noted an expansion of immature CD56^{bright} NK cells accounting for approximately half of all NK cells. Consistent with the mouse data, these cells failed to produce IFNγ in response to IL-2 or IL-15 stimulation whereas they responded normally to IL-12. On the other hand, their capacity to exhibit cytotoxic activity towards target cells was not compromised by the *IR2RB* variant. Nevertheless, it is likely that these differences are explained by the hypomorphic nature of

the variant in comparison to the KO situation in mice rather than reflecting true biological differences. A possible explanation for the CD56^{bright} NK cell expansion is offered by Fehniger *et al.* who incubated CD34+ haematopoietic stem cells in the presence of low-dose IL-2 and observed a preferential differentiation into NK cells rather than T cells[177]. However, the maturation of NK cells usually seen in the face of CMV acquisition was abnormal in cells from Patient 1 as evidenced by the failure to downregulate the TF PLZF and its downstream targets SYK and FcεRIγ as well as to conversely upregulate CD57. In recent years, it became clear that NK cells, albeit as being regarded part of the innate immune system for decades, are educated during development, possess antigen-specific receptors, undergo clonal expansion during infection and generate long-lived memory cells[178].

With NK cells paralleling CD8$^+$ T cell development and differentiation in many aspects, it is not surprising to find a similar lack of terminally differentiated CD8$^+$ T cells expressing CD57 in Patient 1. Given the presence of early T cell memory subsets like T_{CM} it is tempting to speculate that antigen-induced differentiation might be initiated normally but late memory subsets like T_{EM} or T_{TE} might not survive in the periphery. The increasing expression of IL2Rβ during T cell memory differentiation together with the downregulation of IL-7 receptors offers a potential explanation as to why the survival of late memory subsets might be impaired in IL2Rβ deficiency. Due to the very limited amount to patient material, it was not possibly to perform an extensive T cell memory phenotyping in additional IL2Rβ-deficient subjects or to analyse transcriptional programs comprehensively.

It is further difficult to delineate the potential specific contributions of IL-2 and IL-15 to the observed phenotype. It has been shown in mice that both IL-2 and IL-15 induce amino acid uptake and protein synthesis in antigen-activated T cells. Interestingly, IL-2 appeared to be much more potent than IL-15 and the authors relate this to a stronger phosphoinositide 3-kinase (PI3K) activation by IL-2 [179]. This enhanced PI3K activation resulted from positive feedback amplification with increased CD25 expression following IL-2 stimulation[179]. Conversely, exposure to IL-15 is known to negatively impact high-affinity IL-15 receptor expression thereby limiting IL-15 responsiveness of T cells[180]. Since IL-15 mostly acts in a cell-contact dependent manner, it is believed to function mainly as an early indicator of tissue distress reaching much higher concentrations than IL-2 locally[181]. In this view, differences between IL-2 and IL-15, which induce largely overlapping transcriptional programs, are due to their diverging spatial and temporal expression in body tissues[182].

4.1.3 CMV disease in the absence of a functional IL-2 receptor

Given the substantial alterations of the T cell memory compartment seen in IL2Rβ deficiency, a broad susceptibility to infectious agents might have been anticipated. Additionally, *Il2rb* KO mice showed a severe T cell proliferation defect in response to both antigen and mitogens and challenge with different viruses did not result in a pathogen-specific T cell response.

It was therefore surprising to learn that in 6 out of 7 IL2Rβ-deficient subjects, clinically relevant CMV disease, not just CMV viraemia, was reported[126], [147]. CMV organ manifestations included pneumonitis, uveitis, colitis, and hepatitis, although direct evidence from biopsy materials was not obtained in all cases. CMV is a β-herpesvirus, which establishes a latent infection that generally remains asymptomatic in immune-competent hosts but can cause serious illness in immune-compromised individuals. The long-term control of CMV requires considerable effort from

the host immune system and, as already mentioned, relies critically on NK and $CD8^+$ T cells[143], [183].

Kuijpers et al. reported a young infant with IL-7 receptor SCID, who was able to control CMV viraemia by expanding NK cells in the absence of T cells thereby illustrating the importance of NK cell responses to CMV[184]. The cytotoxic T cell compartment of healthy CMV-positive individuals also shows distinctive alterations: up to 30% of $CD8^+$ T cells have been found to be CMV-responsive in healthy adults[185]. These expanded CMV-specific $CD8^+$ T cells show an T_{EM} phenotype, which clearly separates them from other virus-specific T cells arising in the context of *e.g.* chronic EBV or Human immunodeficiency virus infection[186]. This is in line with the observation that EBV viraemia, which was found in 4 of 8 patients, remained asymptomatic and did not cause complications like lymphoma, HLH or smooth muscle tumour development. However, given the often-fatal course of IL2Rβ deficiency, the observation period is rather short and thus not sufficient to rule out a potential, additional susceptibility to EBV disease.

When comparing different T_{reg}opathies with regard to their infectious complications, I noted that this rather selective herpes viral susceptibility to CMV is shared with CD25 deficiency. In 3 out of the 4 patients, were information on encountered pathogens is available, CMV pneumonitis or colitis had been diagnosed[51]–[54]. On the other hand, only 10% of classical IPEX-patients face CMV-associated problems[50] and CMV disease has not been reported in STAT5B deficiency. Whereas detailed information on the $CD8^+$ T cell memory compartment of different T_{reg}opathies is sparse, NK cells have been studied in a patient suffering from CD25 deficiency[187]. In this girl, a NK cell maturation defect with consecutive expansion of $CD56^{bright}$ NK cells and reduced IFNγ production was described, closely mirroring the NK cell phenotype seen in our IL2Rβ-deficient Patient 1. Interestingly, the accumulation of immature NK cells was not found in a STAT5B-deficient subject studied alongside[187]. However, a recent study reported a defective terminal maturation of NK cells in homozygous STAT5B deficiency alongside an impaired IFNγ production in $CD56^{bright}$ NK cells[188]. Given the exclusive involvement of CD25 in IL-2, but not IL-15 signalling, it appears that a functional IL-2 receptor is needed to mount an effective immune response to CMV. Although these related monogenic disorders offer an excellent opportunity to disentangle the anti-CMV contributions of different cytotoxic lymphocyte populations and to provide new and potentially generalizable knowledge about CMV immunity in humans, the shortage of primary patient cells precluded further studies in this direction.

4.2 Importance of fine-tuned JAK-STAT signalling

4.2.1 Altered STAT5B activity leading to immune dysregulation

It is striking to see how both hypomorphic as well as activating variants in *STAT5B* are leading to immune dysregulation, albeit with different flavours. The shared clinical picture of severe eczema is accompanied by autoimmunity and lung disease in LOF situations whereas in GOF patients eosinophilia and allergic manifestations seem to stand out. This highlights the important role of the transcription factor STAT5B in human immunity, however, the limitations of such a statement need to be clarified. The number of patients reported with both LOF but especially GOF variants in *STAT5B* is extremely small and given the overall poor genotype-phenotype correlation in IEI, no firm conclusions about the disease phenotypes are to be drawn as of yet. In addition, no activating germline variants in *STAT5B* have been reported, making the comparison even more complicated.

Nevertheless, the dichotomy of *STAT5B* associated diseases resembles the situation in other members of the STAT family. Monoallelic germline GOF variants have been described in *STAT1* and *STAT3* again leading to complex autoimmune manifestations[11], [64]. Additional somatic activating *STAT6* variants occur in follicular lymphoma[189]. The various ways by which the net gain of transcriptional activity is conferred are best studied in the case of STAT3. Jägle and colleagues investigated *STAT3* variants leading to delayed dephosphorylation and strong basal transcription, enhanced STAT3 responsiveness or increased DNA-binding affinity[190]. In *STAT1* GOF variants, in addition to impaired nuclear dephosphorylation an increased STAT1 protein concentration has been documented[10], [191]. Further studies are needed to delineate the mechanism by which the *STAT5B* p.N642H variant increases transcriptional activity.

Conversely, biallelic loss of function variants are known in the case of STAT1 and STAT2[22], [171]. Here, strongly reduced or absent protein expression causes the lack of signalling capacity and consequent susceptibility to infections. With regard to *STAT3*, KO experiments revealed embryonic lethality in mice and biallelic loss of expression variants are thought to be incompatible with human life as well[192]. However, dominant-negative, heterozygous mutations underlie autosomal-dominant Hyper-IgE syndrome (HIES) characterized by mucocutaneous and pulmonary infections, dermatitis and connective tissue abnormalities[193], [194]. Coming back to the homozygous hypomorphic *STAT5B* variant seen in Patient 2, the analogy to *STAT3* might offer an explanation as to how a missense mutation in the coiled-coil-domain (CCD) might affect phosphorylation. It has been shown that a single amino acid exchange within the CCD of STAT3, through intramolecular interactions, is crucial for IL-6–induced recruitment of STAT3 to the IL-6 receptor and subsequent phosphorylation, nuclear translocation, and DNA binding[195].

In summary, well-balanced STAT5 activity is crucial to prevent immune dysregulation in humans. However, important questions are still unresolved. What is the role of STAT5A in STAT5B deficiency states? Might a potentially tissue-specific capacity of STAT5A to compensate for the loss of STAT5B activity determine the organ manifestations in STAT5B deficiency? Identifying individuals with defective STAT5A function might be needed to finally address this question. How the altered activity of one STAT affects the regulation of residual STATs is also incompletely understood. The CMC occurring in AD-HIES for example is thought to result from unrestrained STAT1 signalling thereby mimicking STAT1 GOF disease. How these complex interactions are regulated on the molecular level, however, is still a matter of debate. Obtaining further insight seems particularly important since specific inhibitors of STAT3 and STAT5 activity are investigated as potential anti-cancer drugs[196].

4.2.2 Disease states associated with aberrant type I IFN signalling

Whereas in the previous section I had discussed signalling imbalances caused by distinct genetic alterations within the same molecule, the following section is dedicated to disturbances of a pathway, namely type I IFN signalling.

In our Patient 4, suffering from *IFNAR1* deficiency, the complete loss of type I IFN responsiveness led to severe inflammatory disease temporally associated with the first application of the live-viral vaccine MMR. Although we failed to detect replication of vaccine strain viruses in patient material, circumstantial evidence and the absence of an alternative explanation prompted us to suspect the illness of Patient 4 to be virally driven. Lately, a few individuals with biallelic loss of expression variants in *IFNAR1* were reported. Hernandez *et al.* had described the first two cases of *IFNAR1* deficiency in 2019[111]. A boy, born to consanguineous parents, developed encephalitis post

MMR-vaccination with detection of measles of PCR in both blood and CSF. However, he recovered and did not experience further invasive infections until the age of 9 years. His younger sister, conversely, died four weeks post MMR-vaccination, but genetic material was not available for sequencing[111]. The other patient carried compound heterozygous variants in *IFNAR1* and developed viscerotropic yellow fever following vaccination at the age of 12 years. In her case, yellow fever vaccine-strain virus was identified in blood samples. She also recovered and remained healthy during the following year. Notably, this girl had received two doses of the MMR vaccine as well as other live-attenuated vaccines without adverse events[111]. Another large consanguineous family was recently reported to carry a large deletion within the IFNAR1 gene[197]. The index patient, who had encountered MMR vaccine without major problems, succumbed to *Herpes simplex virus 1* encephalitis at the age of 20 months, while her cousin, albeit not genetically tested, had died four weeks after MMR vaccination. A third member of the family, also homozygous for the *IFNAR1* deletion, experienced two episodes of aseptic meningitis as an infant and developed hearing loss following *mumps virus* infection at the age of 14. He had not been MMR vaccinated. Very recently, two additional patients with life-threatening *severe acute respiratory syndrome coronavirus 2 (SARS-CoV2)* pneumonia were identified[198]. IFNAR2, the other subunit of type I IFN receptor, had been found mutated in a boy also developing fatal encephalitis following MMR vaccination. Interestingly, his younger sister, in whom MMR vaccination was withheld, has not experienced severe viral disease as of yet[110].

Taken together, of the nine proven or strongly suspected cases of homozygous *IFNAR1* deficiency known to date, five became symptomatic following live viral vaccination. The predominance of MMR-induced illness is likely due to the fact that MMR is the first live attenuated vaccine administered intramuscularly. However, the first live viral vaccine children are exposed to, at least in the western world, is the oral *Rotavirus (RoV)* vaccine. Although it is unclear whether any of the IFNAR1-deficient children has actually received *RoV* vaccination, it is interesting to note, that RoV vaccine induced clinical disease has so far only been reported in children with SCID[199]. This raises the question as to whether viruses encountered at mucosal surfaces might be effectively restrained by type III IFN in these individuals highlighting on the one hand at least partial redundancy of type I and III IFNs at barrier sites? Might these patients without a functional type I IFN response therefore be particularly susceptible to parenterally injected live viral vaccines? Although the mechanisms of attenuation through serial passage in cultured cells are only incompletely understood, they clearly involve the loss of viral type I IFN-antagonists thereby supporting this notion[85]. Contrariwise, the different natural viruses including *SARS-CoV2, HSV-1* and *mumps* isolated in IFNAR1-deficient patients with severe disease argues against a particular, but rather general susceptibility to live-attenuated and natural viruses. However, more patients are needed to answer this outstanding question.

Regarding the HLH seen in Patient 4, it is hard to decipher its relationship to dysregulated inflammatory signaling and potential viral replication. Sterile, HLH-like autoinflammation is increasingly recognized in patients with defective type I interferon signalling involving IFNAR2[200], STAT1[201], STAT2[106] and IRF9[109]. This points towards an essential role of type I IFN in the regulation of inflammation, which is most likely beyond simply restricting viral replication. Indeed, type I IFNs negatively influence various cytokine pathways including IL-1β[202] and IL-17[10].

It is striking to see, that massively dysregulated immune activation, culminating in the identical clinical presentation of HLH, can also be caused by uncontrolled, exaggerated type I IFN signalling as evidenced by Patient 5 and his younger brother, both carrying the mutant STAT2^{R148W} protein[125]. Different from known activating variants in other STAT proteins, this *STAT2* variant

confers GOF behaviour not through inherently increased transcriptional activity, but via resistance to negative-feedback inhibition. This explains why the *STAT2* variant is only pathogenic in the homozygous, but not the heterozygous state. The essential role of the STAT2 residue 148 for the interaction with USP18 is further corroborated by the report of a another family with fatal autoinflammation, in which a homozygous *STAT2* variant resulted in a p.R148Q change[203].

With both insufficient as well as excessive type I IFN activity resulting in immune dysregulation, it becomes clear that a correctly calibrated type I interferon response is crucial to maintain immune homeostasis. The following part of my dissertation discusses molecular mechanisms involved in immune dysregulation as a consequence of perturbed type I IFN signalling.

4.3 Inflammation resulting from aberrant type I IFN signalling responses

4.3.1 Delayed and prolonged IFNAR signalling in ISGF3-deficient cells

The starting point of this project was slightly different from the previous ones in that it was an experimental observation and not an unusual patient phenotype that caught our interest. The finding of prolonged STAT1 phosphorylation in the absence of STAT2 had not been described previously in humans. However, when searching the literature, I came a across an article by Zhao and colleagues, in which they described the upregulation of MHC class II on the surface of STAT2-deficient murine macrophages treated with type I IFN[204]. However, they had attributed the observed prolonged STAT1 phosphorylation to grossly reduced SOCS1 induction. Interestingly, in addition to increased MHC class II expression, delayed IRF1 transcription peaking after 24 hrs of IFNα stimulation and reaching levels comparable to those following IFNγ treatment was noted. Such increased IRF1 transcription compared to control cells following 10 hrs of IFNα exposure had also been found in the initial report of STAT2 deficiency[171]. With both MHC class II and IRF1 being classical IFNγ target genes and IFNγ in turn known as the key cytokine driving HLH pathology[205] I looked for inflammatory manifestations reported in STAT2-deficient human subjects. In seven out of nine patients, summarized by Moens *et al.*, febrile syndromes or severe febrile illness suspected to be virally driven had been reported[105]. Given these interesting aspects it seemed worthwhile to further investigate a potential immune dysregulatory component in these patients.

Since STAT2 is lacking a DNA binding domain and relies on IRF9 to exert its transcriptional effects I also checked the clinical phenotypes of known IRF9-deficient subjects. Up to now, only three IRF9-deficient individuals have been reported. The first child described experienced recurrent fever episodes from the age of five months onwards and was originally suspected to suffer from an auto-inflammatory disorder[108]. While no pathogens were isolated during the febrile episodes, increased levels of TNFα and IL-1β were found in blood. Two weeks after MMR vaccination, the girl was first admitted to the pediatric intensive care unit (PICU) with biliary perforation and disseminated intravascular coagulation. Two months later, suspected septic shock, again without identification of a pathogen, led to another PICU admission before at 23 months of age, *Influenza A virus* infection resulted in in acute respiratory distress syndrome requiring mechanical ventilation. In the other IRF9-deficient family, the index patient experienced four prolonged PICU stays due to septic shock episodes in which again a causative pathogen could never be identi-

fied[109]. We were fortunate to receive primary cells from the latter family and could prove prolonged phosphorylation of both STAT1 and STAT2 in response to type I IFN stimulation. Of note, in *Irf9*-KO murine mixed glial cell culture models consisting of astrocytes and microglia, the macrophage equivalents of the central nervous system, ongoing STAT1 and STAT2 phosphorylation was observed after 12 hrs of IFNα stimulation[206].

Abdul-Sater *et al.* studied murine macrophages with knockouts in either *Stat1*, *Stat2*, or *Irf9* genes together in the context of IFN induced resistance to *Legionella pneumophila* infection[207]. They observed ongoing STAT2 phosphorylation in STAT1$^{-/-}$ macrophages and *vice versa*. When analysing human STAT1-deficient cells, kindly provided by Fabian Hauck, we detected similarly prolonged phosphorylation until 48 hrs of IFNα treatment thereby confirming a defect of negative regulation in all patient cells lacking one of the subunits of the ISGF3 complex. Whereas the failure to induce an antiviral state in all of these patients has been well appreciated, an additional defect in switching this aberrant response to type I IFN off again had thus far not been studied in humans.

Investigating the mechanism underlying the failure of negative regulation, I benefitted from the previous work on STAT2 GOF patients centred around the indispensable role of USP18 in desensitizing IFNAR signalling. The previous hypothesis of insufficient SOCS1 induction leading to ongoing STAT phosphorylation is to be dismissed for several reasons: Despite the lack of rapid SOCS1 induction in all of these cell lines, at later time points, enhanced SOCS1 transcript levels were noted in all ISGF3-deficient human EBV-LCLS lines as well as in all ISGF3-deficient murine macrophages[207]. Therefore, one could expect the delayed SOCS1 upregulation to reach sufficient levels in order to suppress ongoing IFNAR signalling at some later time point. In fact, we observed no signs of desensitization as late as 48 hrs after the onset of stimulation. Further experimental evidence corroborating the key role of USP18 is provided by Taylor and co-workers: both in human monocyte-derived macrophages where *USP18* has been knocked down as well as in iPSC-derived macrophages with *USP18* knockout, prolonged STAT1 and STAT2 signalling were observed after stimulation with type I IFN for 18 hrs[208]. However, the missing capacity of SOCS1 to terminate IFNAR signalling is best illustrated by comparing the IEIs USP18 deficiency and SOCS1 haploinsufficiency. Individuals, who lack USP18 function, display unrestrained type I IFN signalling leading to a phenotype of severe interferonopathy already starting antenatally[120]. Individuals with heterozygous LOF variants in *SOCS1*, conversely, present with early autoimmune features like cytopenias or psoriasis not suggestive of being type I IFN driven but rather indicative of the multifaceted role of SOCS1 acting on various pathways including IL-2 signalling [209].

4.3.2 Inflammatory state of STAT2$^{-/-}$ and IRF9$^{-/-}$ cells mimicking IFNγ stimulation

Having observed a prolonged induction of IRF1 and HLA-DR in STAT2- and IRF9-deficient EBV-LCLs corresponding nicely to the data derived from mice, we thought of a better model to study inflammatory reactions within the human immune system. Macrophages are specialized cells of myeloid origin, which reside in almost every organ and are therefore placed at the forefront of tissue immunity. They phagocytose and degrade dead cells, debris, and foreign material and orchestrate inflammatory processes by acting as antigen-presenting cells and producers of inflammatory cytokines like TNFα, IL-6 or IL-1[210]. Their crucial role in HLH development is reflected by the presence of activated macrophages engulfing haematopoietic cells in lymphatic

tissues being one of the key diagnostic criteria[31]. The essential role of IFNγ in driving macrophage activation was discovered in mouse models of familial HLH. In Perforin-deficient mice, activated cytotoxic T cells, unable to kill their infected targets, alternatively secrete huge amounts of IFNγ thereby driving excessive macrophage activation[205]. However, HLH could also be induced by viral infection in mice with additional disruption of the *Ifng* gene highlighting the presence of alternative pathways involving IL-33 in HLH pathogenesis[211]. This is in line with the description of two unrelated patients with homozygous variants in one of the two subunits of the IFNγ receptor each developing HLH in the context of mycobacterial infection[212]. These two patients, together with two STAT1-deficient individual suffering from HLH[167], [201], substantiate the notion that HLH should be conceptualized as a phenotype of critical illness characterized by toxic activation of immune cells from different underlying mechanisms[213]. Accordingly, we analyzed STAT1$^{-/-}$ iPSC-derived macrophages mainly as a negative control for IFNγ-treatment.

Studying the signalling kinetics in response to IFNα treatment in iPSC-derived KO macrophages I observed results consistent with those obtained in patient-derived EBV-LCLs. Following the hypothesis that inflammatory consequences in STAT2- and IRF9-deficient subjects might be mediated by STAT1-homodimers mimicking IFNγ-stimulation, the iPSC-macrophages were stimulated with IFNα or IFNγ for 48 hrs. Interestingly, we found the baseline transcriptomes of STAT2$^{-/-}$ and IRF9$^{-/-}$ macrophages indistinguishable from each other and clearly different from both WT and STAT1$^{-/-}$ macrophages. Transcriptional responses to IFNα-treatment in STAT2$^{-/-}$ and IRF9$^{-/-}$ revealed a shift from type I to type II IFN triggered output, which was reflected by increased expression of macrophage activation markers as well as increased production of TNFα.

Our data confirm the transcriptional shift towards an IFNγ-like response to be dependent on the presence of STAT1. It was somewhat surprising to see the transcriptional profile of STAT1$^{-/-}$ macrophages treated with IFNα being similar to that of WT cells and in line with this, some expression of antiviral proteins like MX1, RSAD2 and ISG15 was noted. In murine Stat1$^{-/-}$ macrophages, a similar delayed upregulation of *MX1* and *ISG15* transcripts, absent from STAT2- and IRF9-deficient cells, had been noted earlier[207] and attributed to a complex containing IRF9 and STAT2 that can bind a subset of ISRE sites[168], [169], [214]. Furthermore, retained although low level USP18 expression in our STAT1$^{-/-}$ cells is consistent with residual negative regulation of IFNAR signalling despite the lack of ISGF3. STAT1 deficiency therefore could serve as a model to study HLH pathogenesis in the absence of any IFN activity, but this is beyond the scope of both the project and my PhD thesis.

In summary, the data outlined above clearly demonstrate dysregulated and aberrant IFNAR signalling in STAT2$^{-/-}$ and IRF9$^{-/-}$ cells in response to IFNα. Type I IFN is induced in response to viral infection or live-attenuated viral vaccination and is pivotal to innate immune antiviral restraint. A failure to contain viral replication, well-documented for example in STAT2 deficiency[105], might provoke excessive and prolonged type I or type III interferon production, in turn driving aberrant IFNγ-like inflammatory responses. Although paradoxical at first sight, these data provide a potential rationale to use JAKinibs to terminate the dysregulated type I interferon response in order to limit immunopathology in STAT2- and IRF9-deficient individuals. However, the already existing susceptibility to viruses in these conditions is likely to get worse with JAKinib treatment thus requiring a high level of precaution. Whether using JAKinibs as a standard therapy in patients with HLH might improve the still limited 5-year survival of 60% is currently a matter of debate[215].

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This work is dedicated to my wife Hannah and in loving memory of my father.

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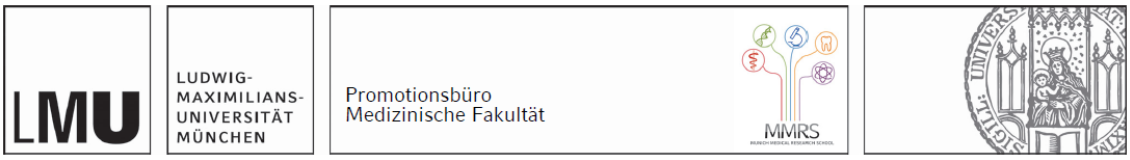

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