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# Functional characterization of a novel IL2RG mutation causing atypical SCID

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## Dedication

*I dedicate this work to my brothers, Franz and Paul.*

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## List of abbreviations

| | |
|---|---|
| ADA | Adenosine Deaminase |
| BMT | Bone Marrow Transplantation |
| BTK | Bruton Tyrosine Kinase |
| CBC | Complete Blood Count |
| CDR3 | Complementary Determining Region 3 |
| CID | Combined Immunodeficiency |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CVID | Common Variable Immunodeficiency |
| DNA | Deoxyribonucleic Acid |
| gDNA | Genomic Deoxyribonucleic Acid |
| GvHD | Graft versus Host Disease |
| HSCT | Hematopoietic Stem Cell Transplantation |
| HTRF | Homogenous Time Resolved Fluorescence |
| IL | Interleukin |
| IL2RG | Interleukin-2 Receptor Common Gamma Chain |
| JAK3 | Janus Kinase 3 |
| KREC | Kappa-deleting Element Recombination Circle |
| NGS | Next Generation Sequencing |
| PID | Primary immunodeficiency |
| RNA | Ribonucleic Acid |
| SCID | Severe Combined Immunodeficiency |
| SGR | Somatic Genetic Rescue |
| STAT5 | Signal Transducer and Activator of Transcription 5 |
| TCR | T Cell Receptor |
| TREC | T Cell Receptor Excision Circle |
| XLA | X-linked Agammaglobulinemia |

# 1 Introduction

## 1.1 Primary immunodeficiencies

Primary immunodeficiencies (PID) belong to a heterogeneous group of congenital disorders occurring both in children and adults. Affected individuals show a high susceptibility to opportunistic infections, autoimmunity diseases and malignancies. To date, more than 300 individual PID diseases have been defined, each characterized by a specific genetic abnormality leading to total or partial absence of cell-mediated and/or humoral immunity [1]. Noticeably, molecular genotypes do not always cover the same clinical phenotype. The symptomatic presentation of the same mutation can be highly diverse reaching from life threatening infections to an asymptomatic appearance. Therefore, timely detection is challenging but crucial for these diseases and requires a high scale of attention and specialized diagnostic [2]. A broad variety of monogenetic immune defects cause a heterogenous group of inherited PIDs **(Table 1)**. Precise diagnosis has been enabled due to progressive genetic analyzation methods providing a vast amount of genetic information of numerous primary immunodeficiencies.

As one of the first PID described in the literature in 1952, X-linked agammaglobulinemia (XLA) is caused by a well-studied inborn error resulting in B-cell deficiency [3]. Pathogenic mutations in the Bruton tyrosine kinase (BTK) are responsible for impaired development and function of progenitor B-cells and plasma cells, respectively. This gene is located on X-chromosome (Xq21.3-Xq22) and therefore only males can be affected by this recessive inherited disease. Additionally, autosomal recessive and dominant forms are commonly known which have been associated with mutations in a variety of other genes such as *BLNK*, *TCF3*, *PIK3R1* and *LRRC8A* [4, 5]. They all share a common pattern of total absence or greatly diminished immunoglobulin production which leads to a qualitative and quantitative reduction in antibody response. Early and severe infections are a predictive indicator for the manifestation of XLA and requires prompt diagnostic followed by an immunoglobulin replacement therapy combined

with prophylactic antibiotic treatment [6]. Another well recognized antibody-deficiency disorder is common variable immunodeficiency (CVID). It belongs to a heterogenous group of disorders with low or absent levels of IgG, IgA and/or IgM serum concentration causing poor humoral-mediated responses to immunization. Affected males and females, which can be carrier of this disease equally, are usually diagnosed later in life than patients with comparable immunodeficiency disorders. Characteristic symptoms are recurrent respiratory infections (bronchiectasis) and a higher risk for autoimmune diseases and malignancies (lymphoma), which decisively contribute to morbidity and mortality in these group of patients [7].

**Table 1. Classification of PIDs: examples and clinical presentation. [1]**

| Classification and examples | Clinical presentation |
|---|---|
| *B-cell (humoral) immunodeficiency* | |
| • XLA<br>• CVID | Recurrent respiratory infection with opportunistic pathogens, autoimmune disease and increased risk for malignancy |
| *T-cell (cellular) immunodeficiency* | |
| • INF-γ/IL-12 | Atypical mycobacterial infections |
| *Combined immunodeficiency* | |
| • Wiskott-Aldrich syndrome | Thrombocytopenia, eczema, recurrent bacterial and viral infections; autoimmune disease |
| • DiGeorge syndrome | Infection, hypoparathyroidism, cardiac abnormalities |
| • Hyper-IgE syndrome | Chronic dermatitis, chronic severe lung infections, lung infections |
| • SCID<br>○ $\gamma_C$ deficiency<br>○ ADA deficiency | Severe recurrent opportunistic infections; failure to thrive, diarrhea and rash |

The T cell linage can be the source for innated immunity disorders as well. When humoral and cell-mediated mechanisms are affected at the same time they are classified as combined immunodeficiencies (CIDs). The severest form of all is known as severe combined immunodeficiency (SCID) and will be discussed in the next section. Adenosine deaminase (ADA) deficiency accounts for approximately 10-15% of all CID cases. It is caused by mutations in the *ADA* gene which encodes for a key enzyme responsible for the purine salvage pathways. Subsequently, the accumulation of toxic metabolites affects T- and B-lymphocytes and results in severe lymphocytopenia [8]. Other CIDs include DiGeorge syndrome, Wiskott-Aldrich syndrome, and hyper-IgE syndrome presenting with typical characteristics like recurrent infections and dysregulation of immunity response (autoimmunity) [7].

### 1.2 X-linked severe combined immunodeficiency (X-SCID)

X-linked severe combined immunodeficiency (X-SCID) is a primary immunodeficiency caused by mutations in the interleukin-2 receptor common gamma chain (*IL2RG*) gene. It is located on the long arm of the X-chromosome at position q13.1 [9]. The common gamma chain ($\gamma_c$) encodes for a subunit that is part of various interleukin receptors such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptor. Defects lead to an impaired transition of the $\gamma_C$-JAK3-STAT5 signaling pathway, which is crucial for sufficient IL-2 production and T-cell proliferation. IL-2 binds to a high-affinity receptor complex, which leads to the activation of Janus kinase 3 (JAK3) **(Figure 1)**. By cross-phosphorylating each other the JAK3 phosphorylates signal transducer and activator of transcription 5 (STAT5). At the end of this cascade stand the dimerized pSTAT5 molecules, which in turn translocate into the nucleus and induce T- and NK-cell proliferation and cytokine production [10].

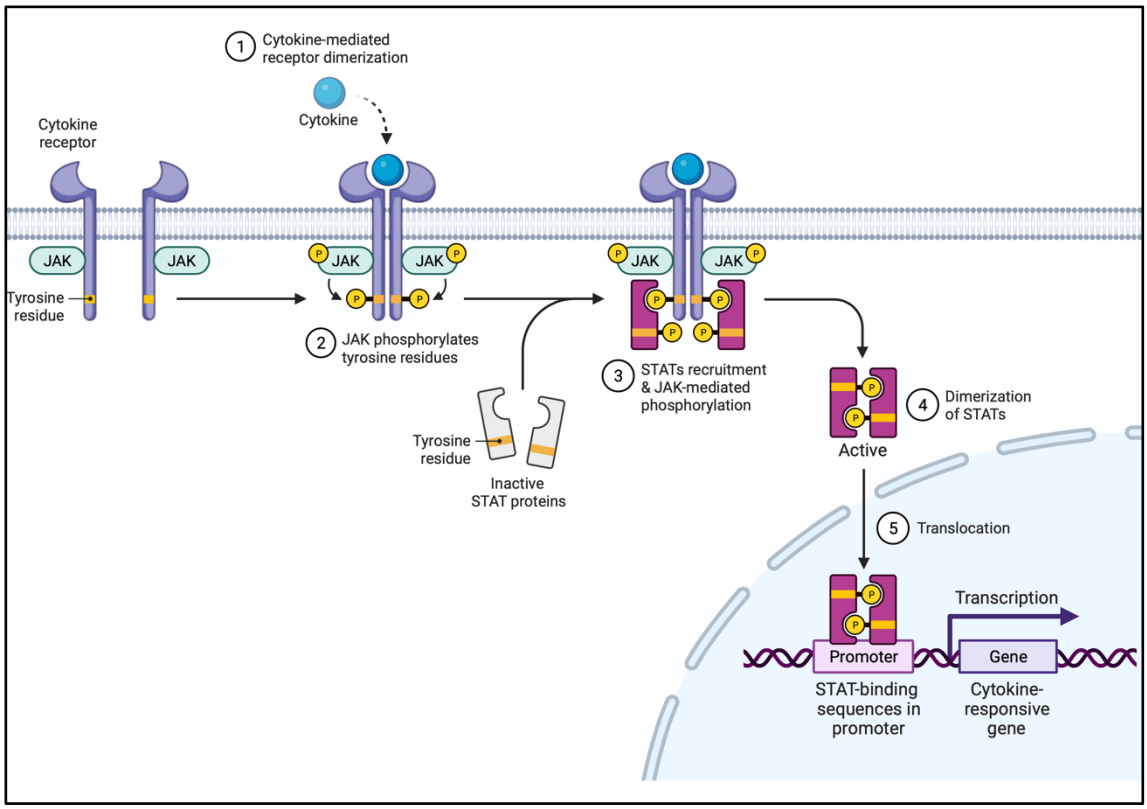



**Figure 1. Intracellular IL-2 receptor $\gamma_C$-JAK3-STAT5 signaling pathway: IL-2 activates JAK3, which in turn phosphorylates STAT5. Finally, T- and NK-cell production is induced (Created with BioRender.com).**

Therefore, X-SCID patients who leak complete gamma chain ($\gamma_C$) function suffer from a total absence of T- and NK-cells. They are highly susceptible to bacterial and viral infections showing in various symptoms like chronic diarrhea, skin rashes and the failure to thrive. Additionally, B cell disfunction and hypogammaglobulinemia are commonly found [11]. Usually, the infants die within the first year of life unless undergoing prompt hematopoietic stem cell transplantation (HSCT) [12]. Although several studies describe successful transplantation, heavy side effects like Graft-versus-Host disease (GvHD) are limiting its application. Alternatively, immunoglobulin-substitution is applied to balance insufficient antibody production [13].

### *1.2.1 Atypical SCID*

Over the last three decades more than 200 hypomorphic *IL2RG* mutations have been published [12, 14-17]. Missense mutations located in the extracellular domain of the $\gamma_C$ make up the vast majority. Other than the extracellular domain (Exon 1-5), the cytoplasmic domain of the receptor is encoded by exons 7 and 8 [18]. The affected patients show milder phenotypes with less severe symptoms and a moderate reduction in T-lymphocytes. Hence, the term “leaky” or “atypical” SCID was introduced. Although a milder phenotype is linked to prolonged survival and lower susceptibility to infections, patients are diagnosed later in childhood or even adulthood because of poorly defined clinical and immunological phenotypes [18]. Additionally, severe cases of X-SCID can be attenuated showing atypical presentation through an event called somatic genetic rescue (SGR), which is described as “natural gene therapy” [19]. It may result in a selective advantage over non-modified cells that is gained by partially or totally reversion of the pathogenic germline mutation. Depending on the cell type and its developmental stage, the process of SGR generates modified clones with a fitness advantage resulting in somatic mosaicism and can be considered as cellular Darwinism [20]. A patient with ADA-SCID was the first one to be identified showing beneficial effects of SGR and it was believed to be a rare phenomenon [21]. However, the development of highly sensitive genetic tools (e.g., NGS) over the past decades contributed to a growing number by detecting such genetic events.

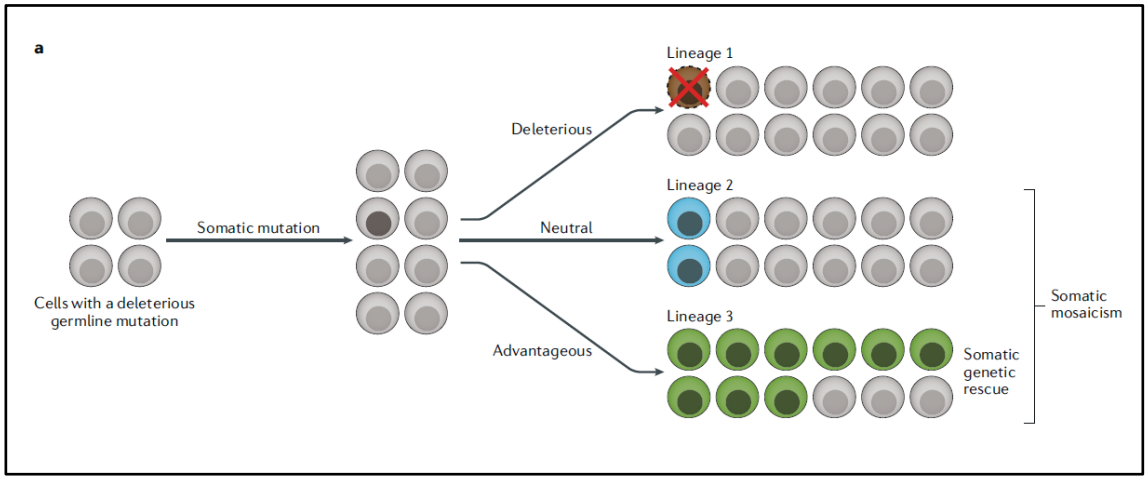



**Figure 2. Somatic mosaicism and its potential effects on cell development (Lineage 1, deleterious; Lineage 2, neutral; Lineage 3, advantageous). Image was adapted from the previous publication [22].**

### 1.3 Diagnostic and conventional treatment

As mentioned above, disease-associated morbidity and mortality are significantly prevented by early diagnosis of SCID. However, more than half of SCID are not detected until adulthood in patients with these disorders, despite the fact that severe symptoms such as recurrent pneumonia or ear, sinus and cutaneous infections have been observed prior to diagnosis [23]. The evaluation of a complete blood count (CBC) including lymphocytes and phagocytic cells usually leads to the first indication of an undiagnosed SCID. To further characterize the suspected immunodeficiency lymphocyte proliferation assays and flow cytometry are important tools which allow the exact enumeration of B-, T-, and NK cells and responsiveness to stimulation [24-26]. These investigations are very effective to identify most cases of SCID although some atypical forms may not be uncovered requiring intensified diagnostic. Aberrations of serum IgG, IgA, IgM, and IgE levels and abnormalities of serum-specific antibody titers may be indicative of immunodeficiencies affecting specifically the B-cell linage [26]. For the confirmation of T cell dysfunction, the performance of stimulation assays with cytokines is a supportive application. For example, impaired intracellular transduction of the interleukin-2 receptor signaling pathway is commonly exanimated by the detection of phosphorylated signal transducer and activator of transcription 5 (pSTAT5) [10]. Eventually, genetic testing verifies clinical and laboratory evidence playing an important role in the initiation of potentially required therapy.

The complexity of the treatment of patients with SCID demands supportive and definitive action that involves aggressive management of ongoing infections, immunoglobulin replacement therapy, and antibiotic and antifungal prophylaxis, respectively [27]. Noteworthy, the application of life attenuated vaccines (e.g., measles/mumps/rubella) is contraindicated in SCID patients due to the risk of disseminated and fatal infections [7]. The standard therapy to effectively treat innate immunodeficiencies underlies the correction of the causative genetic defect. During the past two decades bone marrow transplantation (BMT) or hematopoietic stem cell transplantation (HSCT) was the only promising

approaches in the field of gene therapy. Although excellent long-term survival and long-lasting immune reconstitution were reported in several studies, the finding of HLA-matching donors and the potential risk of the development of Graft-versus-Host disease (GvHD) is limiting its application *in vivo* [28]. Future perspectives are expected to be achieved in the clinical application of novel gene editing tools such as the CRISPR/Cas9 system [13].

### 1.4 Newborn screening

To avoid disadvantageous delay in recognizing SCID on time early identification and intervention are important. According to the clinical manifestation with low or absent T cells, screening newborns for T lymphopenia appears as an ideal strategy for recognizing SCID. However, performing a complete blood count as a screening method to determine lymphocyte numbers proves to be an expensive and time-consuming application [29]. Additionally, its lack of sensitivity led to the consideration of other screening methods. Therefore, the quantification of T cell receptor excision circle (TREC) has been established for newborn screening. TRECs are small, circular pieces of episomal DNA. During the process of T cell receptor rearrangement in naïve T cells these pieces of DNA are cut out of the genome and can be used to make precise statements of thymic emigrants and recent output, respectively [30]. Despite its ideal characteristics of being stable over time, resistant to degradation and replication with subsequent cell division, underlining the significance as a marker for T cell production, it cannot be excluded that infants with other primary immunodeficiencies and secondary causes are falsely identified. In these cases, only clinical and functional characterization including long-term follow up is inevitable.

# 2 Materials and methods

## 2.1 Materials

### *2.1.1 Chemical reagents*

***Table 2: Reagent list***

| Reagent | Company |
|---|---|
| Biocoll separation solution | Biochrom GmbH |
| Nuclease-free water | ThermoFisher Scientific |
| Trypan blue | ThermoFisher Scientific |
| Ethanol | ThermoFisher Scientific |

### *2.1.2 Consumables*

***Table 3: Consumable list***

| Consumables | Company |
|---|---|
| 1.5 – 2mL tubes | Eppendorf |
| 10 – 1000µL filter tips | Tip One |
| 10 – 1000µL tips | Corning Costar |
| 15 and 50mL Falcon tubes | Greiner Bio-One |
| 24, 48 and 96 well-plates | Corning Costar |

### *2.1.3 Liquid cell media and buffers*

***Table 4: Liquid cell media and buffer list***

| Liquid cell media and buffers | Company |
|---|---|
| MACS buffer | Miltenyi Biotec |
| Phosphate Buffered Saline (PBS) | Sigma-Aldrich |
| RPMI 1640 medium | Biochrom GmbH |
| TexMACS medium | Miltenyi Biotec |
| Buffer T1 | Qiagen |
| Buffer B3 | Qiagen |

| | |
|---|---|
| Buffer B5 | Qiagen |
| Buffer BW | Qiagen |
| Buffer PE | Qiagen |
| Buffer PB | Qiagen |
| Permeabilization Buffer A | Miltenyi Biotec |
| Inside Fix | Miltenyi Biotec |

### *2.1.4 Media supplements, growth factors and antibiotics*

***Table 5:***

| **Media supplements, growth factors and antibiotics** | **Company** |
|---|---|
| Interleukin-2 | Miltenyi Biotec |
| Interleukin-7 | Miltenyi Biotec |
| Interleukin-15 | Miltenyi Biotec |
| Penicillin | ThermoFisher Scientific |
| Streptomycin | ThermoFisher Scientific |
| TransAct™ | Miltenyi Biotec |

### *2.1.5 Antibodies and isotypes*

***Table 6:***

| **Antibodies and isotypes** | **Company** |
|---|---|
| Anti-CD3-APC human | Miltenyi Biotec |
| Anti-CD4-VioBright FITC human | Miltenyi Biotec |
| Anti-CD4-APC human | Miltenyi Biotec |
| Anti-CD8-PerCP human | Miltenyi Biotec |
| Anti-CD8-APC human | Miltenyi Biotec |
| Anti-CD19-APC human | Miltenyi Biotec |
| Anti-CD14-APC human | Miltenyi Biotec |
| Anti-CD56-APC human | Miltenyi Biotec |

| | |
|---|---|
| TCR α/β-APC human | Miltenyi Biotec |
| TCR γ/δ-APC human | Miltenyi Biotec |
| Mouse IgG1-PE | Miltenyi Biotec |
| Mouse IgG1-APC | Miltenyi Biotec |
| REA control S-APC | Miltenyi Biotec |
| pSTAT5-PE human | Miltenyi Biotec |

### *2.1.6 Enzymes*

***Table 7: Enzyme list***

| **Enzymes** | **Company** |
|---|---|
| GoTaq DNA polymerase | Promega |
| GoTaq Green DNA polymerase | Promega |
| Proteinase K | Macherey Nagel |

### *2.1.7 Commercial kits*

***Table 8:***

| **Commercial kits** | **Company** |
|---|---|
| NucleoSpin Tissue | Macherey Nagel |
| QIAquick PCR purification | Qiagen |

### *2.1.8 Magnetic separation materials*

***Table 9:***

| **Magentic separation materials** | **Company** |
|---|---|
| $CD3^+$ magnetic beads | Miltenyi Biotec |
| LS columns and magnetic separator | Miltenyi Biotec |

### *2.1.9 Laboratory equipment*

***Table 10:***

| **Laboratory equipment** | **Company** |
|---|---|
| Biological Safety Cabinet | ThermoFisher Scientific |
| Centrifuge Mikro22R | Hettich |
| Centrifuge Rotina420R | Hettich |
| BD FACS Calibur flow cytometer | BD Biosciences |
| Fridge 4°C | Liebherr |
| Freezer -20°C | Bosch |
| Freezer -80°C | Forma Scientific |
| HeraCell Incubator | ThermoFisher Scientific |
| Improved Neubauer cell chamber (hemacytometer) | Marienfield |
| Light microscope | Zeiss |
| MiniSpin centrifuge | Heathrow Scientific |
| Pipettes (10µL, 20µL, 100µL, 200µL, 1000µL) | Brand |
| Thermalcycler C1000 Touch | BioRad |
| Thermoblock | ThermoFisher Scientific |
| Vortex reax 2000 | Heidolph |
| Weight balance E400D | Ohaus 20 |

## 2.2 Methods

### *2.2.1 Patient recruitment*

"The patients were recruited through the outpatient clinic of the Center of General Pediatrics, Oncology/Hematology, at the University Children's Hospital, Tübingen. Clinical data was collected retrospectively. Informed consent for this study was obtained in accordance with the Declaration of Helsinki and the Institutional Review Board approval from the University of Tübingen Ethics Committee." [31]

### *2.2.2 T cell isolation*

Whole blood was taken from all three patients and mixed 1:1 with sterile PBS. 20mL of the mixture was added slowly in 20mL of Biocoll with avoiding of mixing. The sample was then centrifuged at 20°C, 1/1R, 800g for 30 minutes. Afterwards, the PBMCs were taken with rotatory movements at lowest speed with a 5 mL pipette and placed in a 50 mL Falcon. The Falcon was filled up with PBS and centrifuged again at 20°C, 9/9R, 400g for 10min. After discarding the supernatant, the cells were wash with 5 mL PBS and the Falcon was filled up with PBS again. Another centrifugation step (20°C, 9/9R, 400g, 10 min) followed. The supernatant was discarded once again and the pellet was resuspended in 4 mL PBS. 10 µL was taken for cell counting, the rest was filled up with PBS. While counting the cells and calculating the appropriate volume of MACS buffer and $CD3^+$ microbeads (80 µL of MACS buffer and 20 µL of $CD3^+$ beads per $10^7$ cells) the cells were centrifuged at 4°C, 9/7R, 200g for 10 minutes. The supernatant was discarded and the cells were resuspended in the calculated volume of MACS buffer and $CD3^+$ microbeads. The mixture had to be shaken very carefully and subsequently incubated in the fridge for 15 minutes at 4°C. After the incubation time was over, the cells were washed with 1.5 mL of MACS buffer per $10^7$ cells. The next centrifugation step (4°C, 9/9R, 300g for 10 min) was followed by resuspending the cells in 500 µL of MACS buffer per $10^8$ cells. Avoiding the formation of bubbles was crucial for the separation step followed. Before adding the cells to one of the LS columns of the magnetic separation device, a total

volume of 3 mL MACS buffer was applied to the LS column. After application of the cells another 3 mL of MACS buffer was added. After almost everything was eluted two more rounds of this washing step was performed. After the last washing, 5 mL of MACS buffer was added and the LS column removed from the magnetic device. By using the syringe pressure was applied to release the $CD3^+$ T-cells in a new 50 mL Falcon. The Falcon was filled up with 50 mL RPMI medium with no supplements. Three more centrifugation steps at 4°C, 9/7R, 259g for 9 minutes followed. After the second washing the cells were resuspended in 4 mL RPMI and 10 µL was taken for cell counting. Eventually, isolated $CD3^+$ T-cells were resuspended in 1 mL of RPMI per $10^6$ cells.

### *2.2.3 TCR Vβ repertoire*

"PCR high-resolution spectra type analysis of peripheral human TCR repertoires was done as previously described [32]. Shortly, TCRβ sequences were first amplified with a panel of 27 Vβ-gene-specific 5′ primers (Vβ), and a C-gene-specific reverse primer. The amplification products were then fluorescently labeled using an additional 3′ C-gene primer and resulting run-off products identified by length polymorphism on polyacrylamide sequencing gels to reveal precise sizes of amplicons. Appropriate software was used to depict the size and number of peaks corresponding to discrete CDR3 (complementary determining region 3) lengths." [31]

### *2.2.4 Phospho-STAT5 cell-based HTRF assay*

To detect possible STAT5 phosphorylation in patient's T cells a two-plate STAT5-Phosphrylation-Assay protocol for suspension cells was performed. Cells were seeded at $1x10^5$ cells/well in a96-well plate. For stimulation 5 µL of compound (IL-2 10 000 U/mL) was dispensed. After several time course studies of compound treatment optimal stimulation times was determined at 20 min. Supplemented lysis buffer (10 µL) was added to the cells immediately afterwards and incubated for 30 min at room temperature under shaking. Then, homogenization was ensured by pipetting up and down. 16 µL of the lysate had

to be transferred from the 96-well cell-culture plate to a HTRF 96-well low volume white plate. Eventually, the premixed antibody solution (4 µL of d2 and Eu Cryptate) was added. After covering the plate with a plate sealer, the cells were incubated overnight. At the next day the fluorescence emissions were read at two different wavelengths (665 nm and 620 nm) on a compatible Tecan-reader. For the calculation of the HTRF (homogeneous time resolved fluorescence) ratio the following formular was applied: HTRF ratio $= \frac{Signal\ 665\ nm}{Signal\ 620\ nm} x10^4$.

### *2.2.5 Intracellular pSTAT5 flow cytometry staining*

Isolated PBMCs were treated with 100, 1000 and 10 000 U/mL of IL-2 (Miltenyi Biotec) and 1, 10 and 100 ng/mL of IL-7 and IL-15 (Miltenyi Biotec), respectively. Afterwards, the cells were fixed by adding 250 µL Inside Fix (Miltenyi Biotec) to 1 mL cell suspension with $10^6$ cells. The mixture was then incubated for 10 min at room temperature and centrifuged at 500xg for 5 min at 4°C. 1 mL ice-cold (-20°C) Permeabilization Buffer A (Miltenyi Biotec) per $10^6$ cells was slowly added to permeabilize resuspended cells. Tubes were placed on ice for 30 min after being vortexed. First washing step was performed by adding 3 mL of buffer and centrifugation at 500xg for 5 min at 4°C, second washing by adding 4 mL of buffer and centrifugation at 500xg for 5 min at 4°C. Followed by adding 10 µL of anti-pSTAT5 (BD) to resuspended nucleated cells (up to $10^7$ per 100 µL). Mixture was then incubated again for 30 min in the dark at room temperature. Last washing step was done by adding 1 mL buffer and centrifugation at 500xg for 5 min. Eventually, the pellet was resuspended in 100 µL of buffer and mixed well for flow cytometry acquisition.

### *2.2.6 Flow cytometry and cell sorting*

“Various subsets of $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD56^+$, αβ, γδ, and NKT ($CD3^+/CD56^+$) cells were sorted from PBMCs using Flow Cytometry (MACSQuant Tyto , Miltenyi Biotec) with corresponding anti-human antibodies.

Genomic DNA (gDNA) was isolated from peripheral blood and with NucleoSpin Tissue kit (Macherey Nagel) according to the following method description.“ [31]

### *2.2.7 DNA isolation*

Firstly, cells were resuspended in a final volume of 200 µL Buffer T1 followed by adding 25 µL Proteinkinase K solution and 200 µL Buffer B3. The mixture was vortexed and incubated at 70°C for 15 minutes. After the incubation time was over 210 µL ethanol (100%) was added to the sample and vortexed vigorously. The sample was then applied to a NucleoSpin Tissue Column, which was placed into a Collection Tube. Centrifugation was performed for 1 minute at 11 000g. The flow-through was discarded and the column was placed in a new Collection Tube. Subsequently, two washing steps followed. Firstly, 500 µL Buffer BW was added to the column, centrifuged for 1 minute at 11 000g and placed back into the Collection Tube. Secondly, 600 µL Buffer B5 was added to the column, centrifuged for 1 minute at 11 000g and placed back into the Collection Tube. One more centrifugation for 1 minute at 11 000g was performed. Afterwards, the NucleoSpin was placed into a 1.5 mL Eppendorf Tube and 30 µL $H_2O$ was added. Before centrifuging for 1 minute at 11 000g, incubation for 1 minute at room temperature was implicated. At the end, the DNA concentration was measured using NanoDrop software.

### *2.2.8 PCR*

A total volume of 20 µL was achieved by mixing 1 µL Forward and Reverse Primer, respectively, 10 µL of TaqGreen Polymerase and the calculated volume of DNA (100 ng) and $H_2O$. PCR was performed using the following set up: Initialization at 95°C for 2 minutes, denaturation at 95°C for 40 seconds, annealing at 62°C for 30 seconds and extension at 68°C for 1 minute. These steps were repeated 40 times. Last step was a cool down at 4°C.

### *2.2.9 DNA purification*

5 volumes of Buffer PB was added to 1 volume of the PCR reaction product. To bind DNA, the mixture was applied to a QIAquick column, which was placed in a 2 mL Collection Tube. After centrifugation for 1 minute at 17 900g the flow-through was discarded and the QIAquick was placed back in the Collection Tube. To wash, 750 µL Buffer PE was added to the QIAquick column and centrifuged for 1 minute at 17 900g. Discarding the flow-through was repeated once more. To remove residual wash buffer one more centrifugation step ( 1min, 17 900g) was performed. Eventually, the QIAquick column was placed in a clean 1.5 mL microcentrifuge tube accompanied by 30 µL $H_2O$ to be centrifuged one last time for 1 minute at 17 900g.

### *2.2.10 Sequencing and genomic analysis*

PCR products were purified by using QIAquick PCR purification kit (Qiagen) after amplification was performed. For measuring the DNA concentration NanoDrop 2000 was used followed by mixing 75 ng with the proper forward primer and water volume. The samples were sent for Sanger sequencing to Eurofins Genomics whereas the provided results were analyzed with ApEexe software to compare the patient samples with a wild-type readout.

### *2.2.11 Maternal engraftment*

“To exclude the persistence of maternal $CD3^+$ T cells, chimerism analysis was performed by PCR amplification of short tandem repeat (STR) sequences. Several STR loci of maternal and patients’ gDNA from $CD3^+$ T cells samples were amplified by Taq-Polymerase (Qiagen) with different primer pairs (Thermofisher) to obtain at least one informative locus. Detection of patients and maternal DNA was carried out on sequential patient samples using 3130-16 Genetic Analyzer and GeneMapper Software (Thermofisher).” [31]

# 3 Results

## 3.1 Case report and clinical history

“Three brothers were born to nonconsanguineous healthy German parents. Although the clinical symptoms vary in their severity, all of them show signs of increased susceptibility to opportunistic respiratory and viral infections. Patient 1 (P1) was affected the most, suffering from molluscum contagiosum and cutaneous warts on both hands and feet since the age of 6 and 11, respectively. Before the exploration of a genetic mutation at the age of 19, P1 was treated with various therapies to reduce warts. No satisfying improvement has been achieved attempting all kinds of topical and systemic treatments such as cryotherapy, keratolysis, curettage, laser therapy, imiquimod cream, local IL-2 injection and retinoids. Subcutaneous injection of interferon alpha (INF-α) was applied twice within four years, whereas the first therapy cycle showed more improvement by light regression of warts. However, both times the application had to be stopped due to severe side effects (e.g., recurrent fever, hair loss, mental health issues). In addition, the patient developed chronic respiratory infections leading to bronchiectasis after being hospitalized twice due to severe cases of pneumonia. Genetic examination excluded cystic fibrosis (CF) and primary ciliary dyskinesia (PCD). Therefore, some form of cellular immunodeficiency was firstly suspected in P1 at the age of 19.” [31] After detection of massive $CD4^+$ T cell and NK cell lymphopenia and dysgammaglobulinemia he was referred to detailed immunological investigation because of a suspected late-onset combined immunodeficiency disorder. The family history of the patient revealed that his two younger brothers (Patient 2 and 3) also suffered from recurrent warts and molluscum contagiosum since early childhood. “The warts of patient 2 (25 years old) had resolved eventually without specific treatment at the age of 14, whereas Patient 3 (23 years old) is still moderately affected. Moreover, P2 shows no signs of chronic respiratory infections ether, whereas P3 was diagnosed with bronchiectasis and atelectasis after hospitalization because of a severe case of pneumonia at the age of 22 (**Table 11**).” [31]

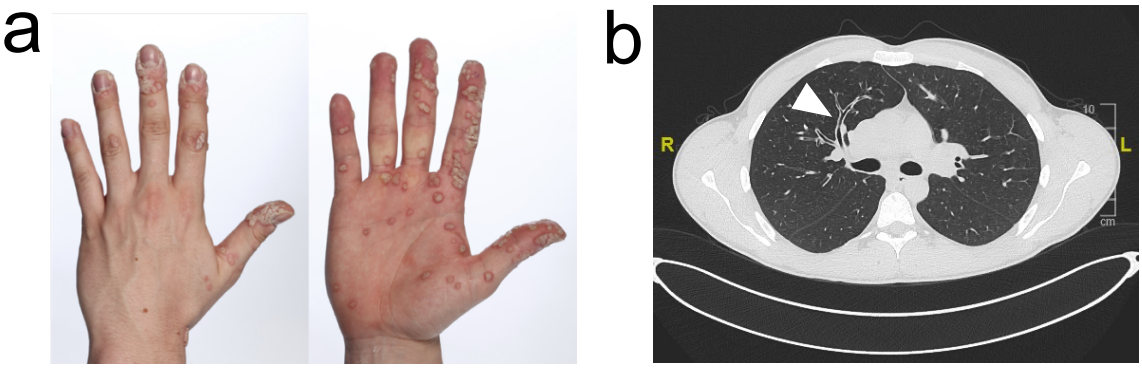



**Figure 3. Clinical phenotype of P1 at presentation: (a) Cutaneous warts on palmar and extensor surface of both hands. (b) High-resolution tomography shows bronchiectasis in the right middle lobe (arrowhead). [31]**

Despite this wide range of symptoms all three brothers commonly share a suspicious $T^{low}B^{+}NK^{low}$- phenotype with dysgammaglobulinemia. Besides slightly increased IgG1 levels, the elevation of IgG subclasses revealed diminished and even undetectable IgG4 levels, respectively, although B-cells count was normal. No similar events are known throughout the family pedigree. Genetic analysis of the siblings and their mother revealed a missense mutation c.458T>C; p.Ile153Thr in exon 4 of the *IL2RG* gene. Accordingly, the asymptomatic mother is the carrier of the inherited X-chromosomal recessive mutation. The genetic findings were confirmed by Sanger sequencing of all three brothers. Based on the genetic knowledge, the milder phenotype and specific blood parameters patient P1, P2 and P3 were diagnosed with a rare form of “atypical” or so-called “leaky” SCID. Only P1 received anti-microbial prophylaxis and immunoglobulin substitution therapy due to his heavy symptomatically appearance. Since following this treatment plan no severe bacterial infection has occurred even though cutaneous warts and bronchiectasis are still present in P1. However, should the clinical condition worsen the patients have already undergone medical briefing on hematopoietic stem cell transplantation (HSCT). In the meantime, careful and frequent monitoring is mandatory because of the uncertain stability of the clinical and cellular immunologic phenotype.

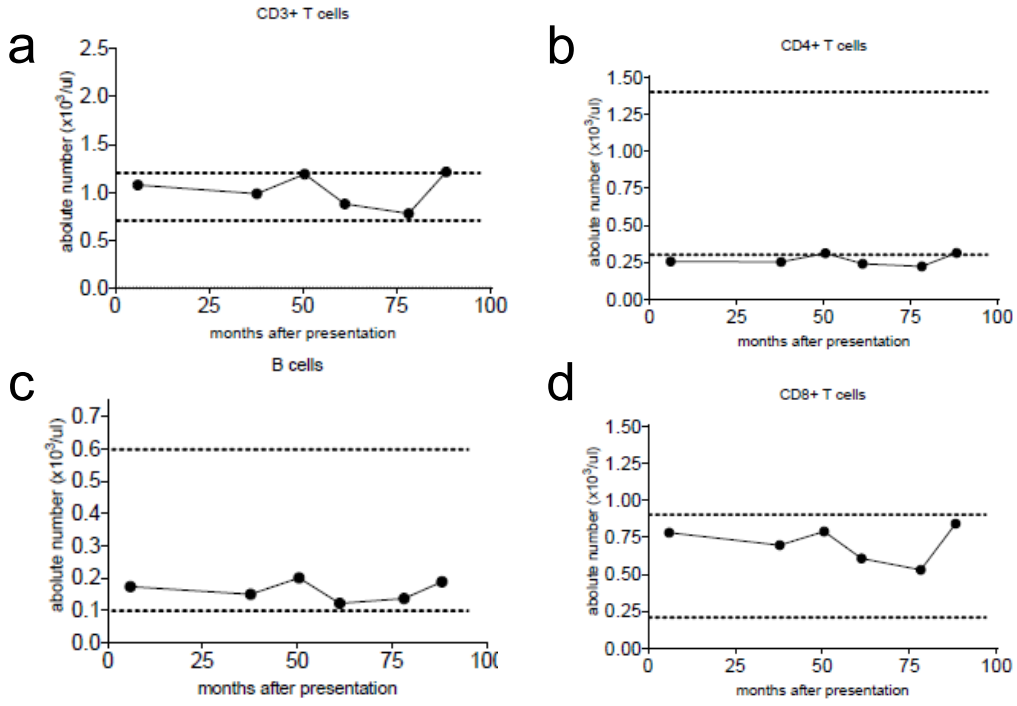
**Table 11. Clinical history of reported patients [31]**

| | P1 | P2 | P3 |
|---|---|---|---|
| Patient (age, ethnicity) | 26-year-old German male | 25-year-old German male | 23-year-old German male |
| Consanguinity | No | No | No |
| Medical history | Viral infections with molluscum contagiosum and cutaneous warts due to *Human papilloma virus*, chronic respiratory infections, two hospitalizations due to severe pneumonia within 7 years, *Pseudomonas aeruginosa* | Viral infections with molluscum contagiosum and cutaneous warts due to *Human papilloma virus*, otitis, susceptible to viral and fungi infections with skin rash | Viral infections with molluscum contagiosum and cutaneous warts due to *Human papilloma virus*, chronic respiratory infections with recurrent sinusitis, hospitalization due to a severe case of pneumonia |
| Family history | No family history suggestive of immunodeficiency, mother detected as conductor of X-chromosomal recessive mutation | No family history suggestive of immunodeficiency, mother detected as conductor of X-chromosomal recessive mutation | No family history suggestive of immunodeficiency, mother detected as conductor of X-chromosomal recessive mutation |
| Clinical phenotype (at diagnosis) | Bronchiectasis and generalized cutaneous warts | Asymptomatic | Bronchiectasis and right middle lobe atelectasis, local cutaneous warts |
| Viral examination | VZV IgG positive<br>Positive EBV PCR<br>Negative CMV PCR<br>Positive HPV 2, 27, 57 PCR | n.a. | n.a. |
| Therapy | Local IL-2 injection<br>Cryotherapy<br>Laser therapy<br>Keratolysis<br>Imiquimod cream<br>Retinoids<br>INF-α<br><br>IVIG substitution<br>Antibiotic prophylaxis | - | Cryotherapy<br>Laser therapy<br>Keratolysis<br>Imiquimod cream |

VZV = varicella-zoster virus; CMV = cytomegalovirus; EBV = Epstein-Barr virus; HPV = human-papilloma virus; PCR = polymerase chain reaction; n.a. = not assessed.

### *3.1.1 Immunological characteristics*

P1 was referred from the Department of Hematology/Oncology at Stauferklinikum Hospital, Schwäbisch Gmünd. Here, low serum levels of IgG immunoglobulin subclasses were detected for the first time. Combined with his clinical presentation (bronchiectasis and generalized cutaneous warts, **Fig. 3**) an undiagnosed form of inherited immunodeficiency was suspected. Therefore, P1 and his brothers, who show similar but attenuated symptoms, were sent to the Center of General Pediatrics, Oncology/Hematology, at the University Children's Hospital, Tübingen for further immunological investigations. The previously observed dysgammaglobulinemia was confirmed in all three patients showing slightly elevated IgG1 (P1 1670 mg/dl; P2 1550 mg/dl; P3 1270 mg/dl) and low IgG2 (P1 79 mg/dl; P2 147 mg/dl; P3 168 mg/dl) and almost undetectable IgG4 (P1 <0.3 mg/dl; P2 0.6 mg/dl; P3 0.4 mg/dl) levels, respectively (**Table 12**). IgA, IgM and IgE immunoglobulins were normal. [31]



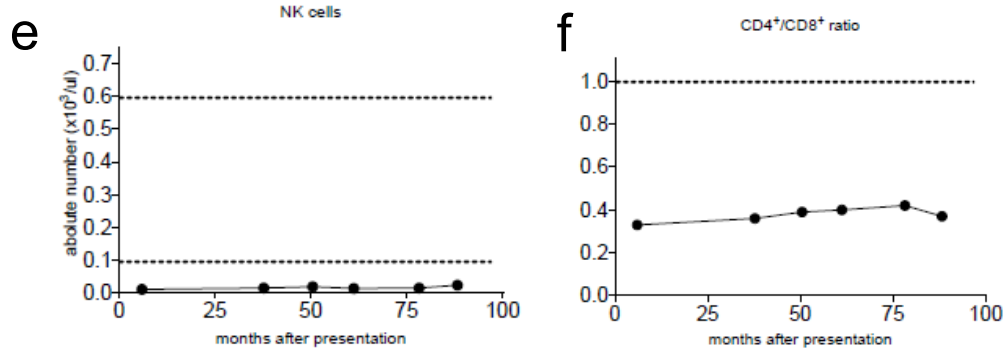



**Figure 4. Long-term immunological status of P1 over 7 years showing the development of (a) $CD3^+$, (b) $CD4^+$, (c) $CD8^+$ T-, (d) B- and (e) NK-cells. (f) illustrates the shifted but steady $CD4^+/CD8^+$ ratio. Dotted lines represent upper and lower limits of normal. [31]**

In addition, the long-term immunological status of P1 was documented over 7 years (**Fig. 4a-f**). Low $CD4^+$ T cells contributed to repetitively reduced lymphocyte counts. Massively decreased $CD56^+$ NK cell numbers combined with a shifted $CD4^+/CD8^+$ ratio (< 1,0) completed the picture of a $T^{low}B^+NK^{low}$-phenotype and strengthened the suspicion of a cell-mediated immunity disorder. Interestingly, his brothers show similar but not as prominent clinical and immunological findings. Therefore, genetic analysis for an inherited immunity disorder was initiated.

### 3.2 Genetic analysis

The corresponding c.458T>C; p.Ile153Thr *IL2RG* mutation in P1, P2 and P3 was detected by Next Generation Sequencing (NGS) at the Department of Human Genetics, Tübingen and has not been described in the literature so far. Because of its X-chromosomal linked inheritance this novel heterozygotic mutation was consequently detected in the patient's mother and she was confirmed as the carrier. Further genetical examination on *JAK3, ILR7, PNP, ZAP70, RFXANK, CIITA, RFX5, FOXN1, RFXAP, CD3D, CD3E, STK4, CD3G, CD247, UNC119* and *CD8A* were performed without any abnormal findings. [31]

**Table 12. General immunological characterization of the affected brothers [31]**

| | P1 | | | | P2 | | P3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **08/2019** | **01/2020** | **05/2020** | **07/2020** | **01/2020** | **05/2020** | **05/2020** | **07/2020** | **Normal range** |
| Leukocytes, absolute/µl | 5710 | 5790 | 6114 | 4610 | 5470 | 5200 | 4700 | 5300 | 3800 - 10300 |
| Lymphocytes, absolute/µl | 1276 | 520↓ | 856↓ | 1011↓ | 1789 | 1456 | 1316 | 697↓ | 1100 - 3200 |
| $CD3^+$, absolute/µl (%) | 866↓ (67,9) | n.a. | 608↓ (71) | 511↓ (50,5) | 1502 (84) | 1165 (80) | 987 (75) | 697↓ (47) | 900 - 4500 |
| $CD4^+$, absolute/µl (%) | 146↓ (11,4) | n.a. | 146↓ (17) | 151↓ (14,9) | 272↓ (15,2) | 233↓ (16) | 197↓ (15) | 178↓ (12) | 500 - 2400 |
| $CD8^+$, absolute/µl (%) | 685 (53,7) | n.a. | 411 (48) | 331 (32,7) | 1073 (60) | 815 (56) | 737 (56) | 430 (29) | 300 - 1600 |
| $CD4^+/CD8^+$ ratio | 0,21 | | 0,35 | 0,46 | 0,25 | 0,29 | 0,27 | 0,41 | > 1,0 |
| $CD19^+$, absolute/µl (%) | 87↓ (6,82) | n.a. | 103↓ (12) | 76↓ (7,5) | 179↓ (10) | 175↓ (12) | 132↓ (10) | 119↓ (8) | 200 - 2100 |
| $CD56^+$, absolute/µl | 153 (12) | n.a. | 21↓ (2,5) | 23↓ (2,3) | 72↓ (4) | 22↓ (1,5) | 61↓ (4,6) | 163 (11) | 100 - 1000 |
| IgG, mg/dl | 1760↑ | 1920↑ | n.a. | 1680↑ | 1550 | 1349 | 1470 | n.a. | 700 - 1600 |
| IgG1, mg/dl | 1670↑ | 1800↑ | n.a. | n.a. | 1550↑ | n.a. | 1270↑ | n.a. | 405 - 1011 |
| IgG2, mg/dl | 79↓ | 181 | n.a. | n.a. | 147↓ | n.a. | 168↓ | n.a. | 169 - 786 |
| IgG3, mg/dl | 63.1 | 73.1 | n.a. | n.a. | 149↑ | n.a. | 55.3 | n.a. | 11 - 85 |
| IgG4, mg/dl | <0.3↓ | 10.9 | n.a. | n.a. | 0.6↓ | n.a. | 0.4↓ | n.a. | 3.0 - 201 |
| IgA, mg/dl | 240 | 269 | n.a. | n.a. | 230 | n.a. | n.a. | n.a. | 70 - 400 |
| IgM, mg/dl | 111 | 114 | n.a. | n.a. | 166 | n.a. | n.a. | n.a. | 40 - 230 |
| IgE, mg/dl | 16 | 19 | n.a. | n.a. | 9 | n.a. | n.a. | n.a. | - 100 |

TCR = T cell receptor; Ig = immunoglobulin; n.a. = not assessed.

### 3.3 Functional characterization

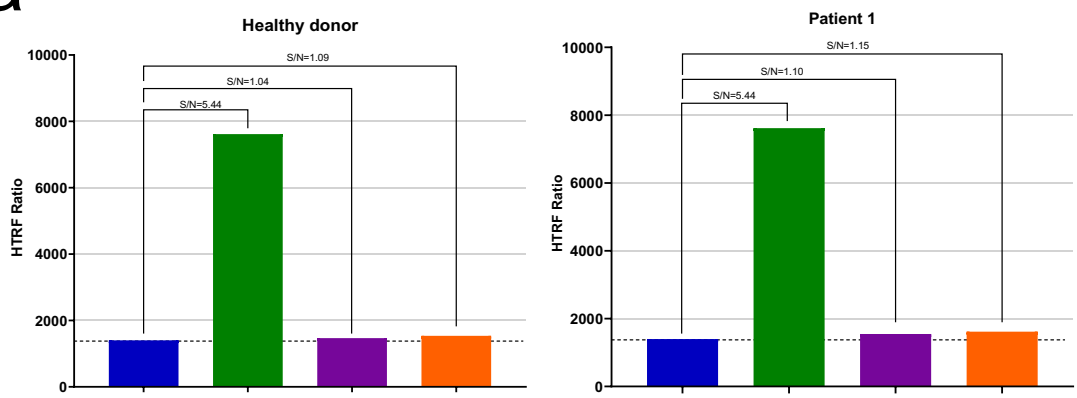
The common gamma chain is an important subunit in cytokine-receptors such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptor as described previously in the text (**1.3, Fig. 1**). Their proper function guarantees for sufficient T- and NK-cell proliferation and cytokine production, respectively. Therefore, patients with *IL2RG* mutations can present with an impaired or total absence of STAT5 phosphorylation, which can be induced e.g., by IL-2, IL-7 and IL-15 stimulation.

Functional pSTAT5 analyzation was performed using two different methods: (1) cell-based HTRF assay and (2) intracellular pSTAT5 staining on flow cytometry. The functional characterization led to further genetic analyzation confirming the correlation between structural and functional T cells deficiency.

#### *3.3.1 Phospho-STAT5 cell-based HTRF assay*

Initially, the $\gamma_C$-pSTAT5-JAK3 signaling pathway of these receptors was analyzed using a novel cell-based HTRF assay (**Fig. 5a-c**). The negative control lysate (blue) served as control. The positive control lysate (green) with a S/N ratio of 5.44; 4.63 and 3.16 showed a significant reaction. However, both healthy donors and patients sample showed no significant HTRF ratio (The S/N of patient 1 = 1.15, patient 2 = 1.18, patient 3 = 1.08. If the S/N of HTRF signal is >1.2, it is considered as significant in the assay).

a



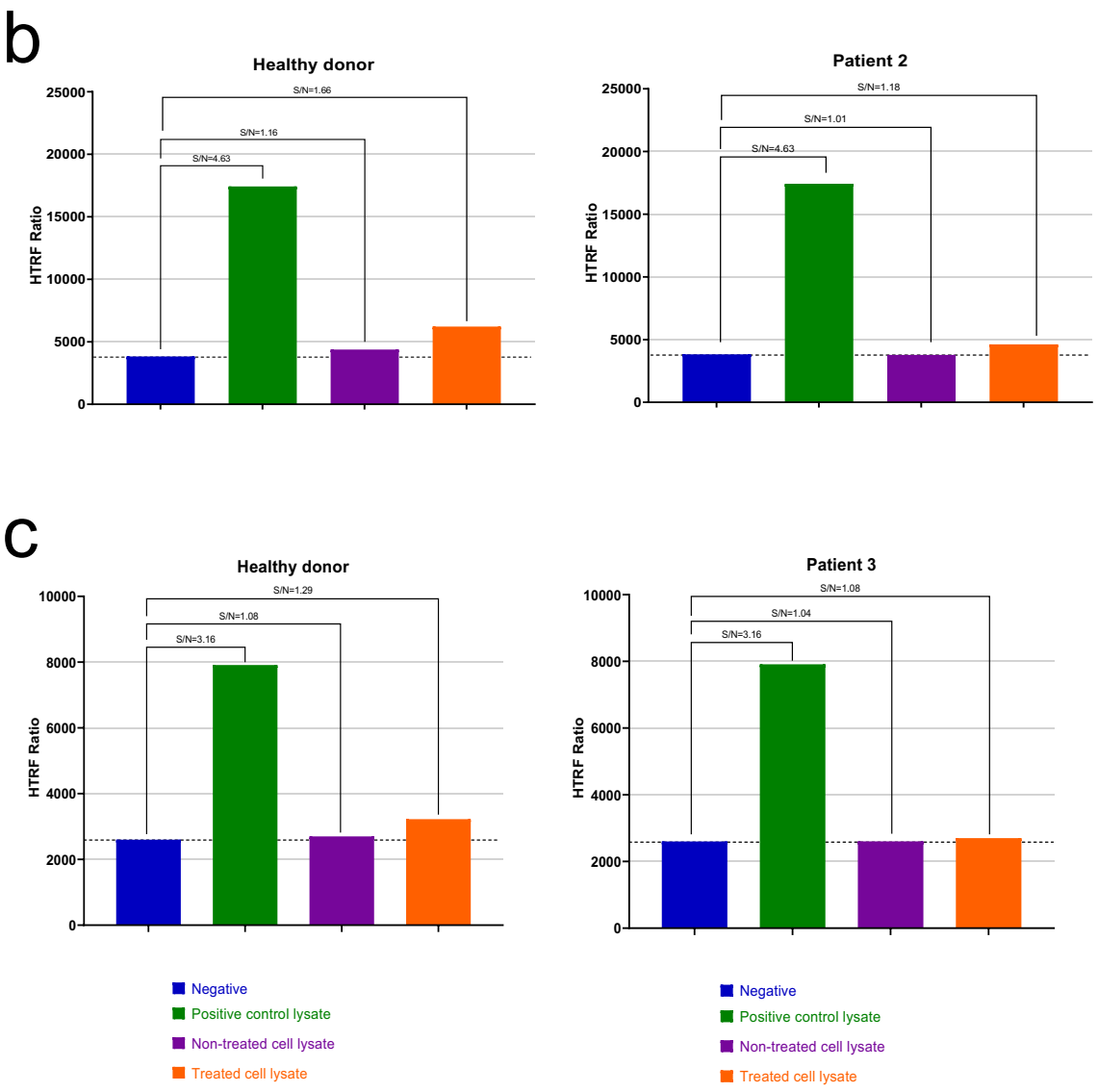



**Figure 5. Phosphp-STAT5 cell-based HTRF assay comparison of (a) P1, (b) P2, and (c) P3 with three age-matched healthy controls. A ratio of >2,0 is considered as significant between negative control (N, blue) and positive control (S, green).**

Multiple rounds of troubleshooting resulted in the adaption of stimulation time, cell density and cytokine concentration. However, no significant differences were observable between treated and non-treated samples. Hence, it was decided to perform intracellular staining on flow cytometry to investigate the difference in STAT-5 phosphorylation between healthy donors and patients.

### *3.3.2 Intracellular Phospho-STAT5 flow cytometry staining*

This time a significant dose-depending activation on $CD4^+$ and $CD8^+$ was detectable. The titration was performed using three different concentrations of IL-2 (100, 1000, 10 000 U/mL), IL-7 and IL-15 (1, 10, 100 ng/mL). The intracellular pSTAT5 staining showed comparable phosphorylation between healthy donors and patients using high doses of IL-2 (1000 and 10 000 U/mL). Interestingly, at low IL-2 concentrations (100 U/mL) a partial activation of pSTAT5 was observed in both patients (**Fig. 6**). To clarify whether IL-7 and IL-15 signaling was also partially impaired the staining experiment was repeated once more using 1, 10, and 100 ng/mL IL-7 and IL-15. However, there was no difference compared to healthy controls (**Fig. 7a,b**).

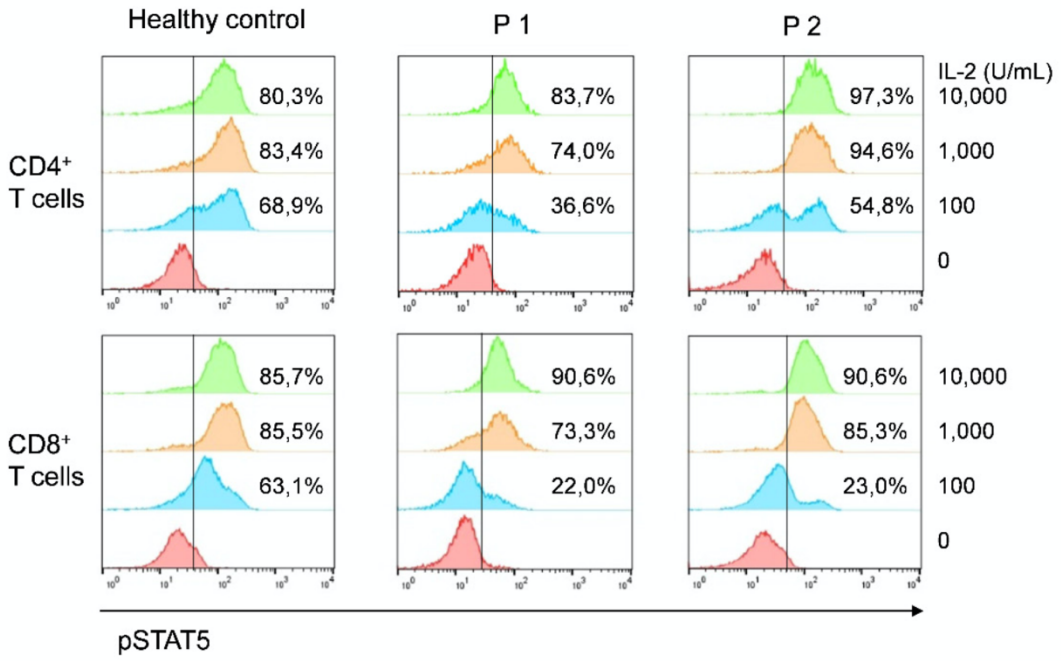



**Figure 6. STAT5 phosphorylation: $CD4^+$ and $CD8^+$ T cells of P1 and P2 exhibited partial $\gamma_C$ signaling in response to stimulation with low concentrations of IL-2 (100 U/mL) compared to healthy donors (n=2). High concentrations (1000 and 10 000 U/mL) showed similar STAT5 phosphorylation to healthy controls. [31]**

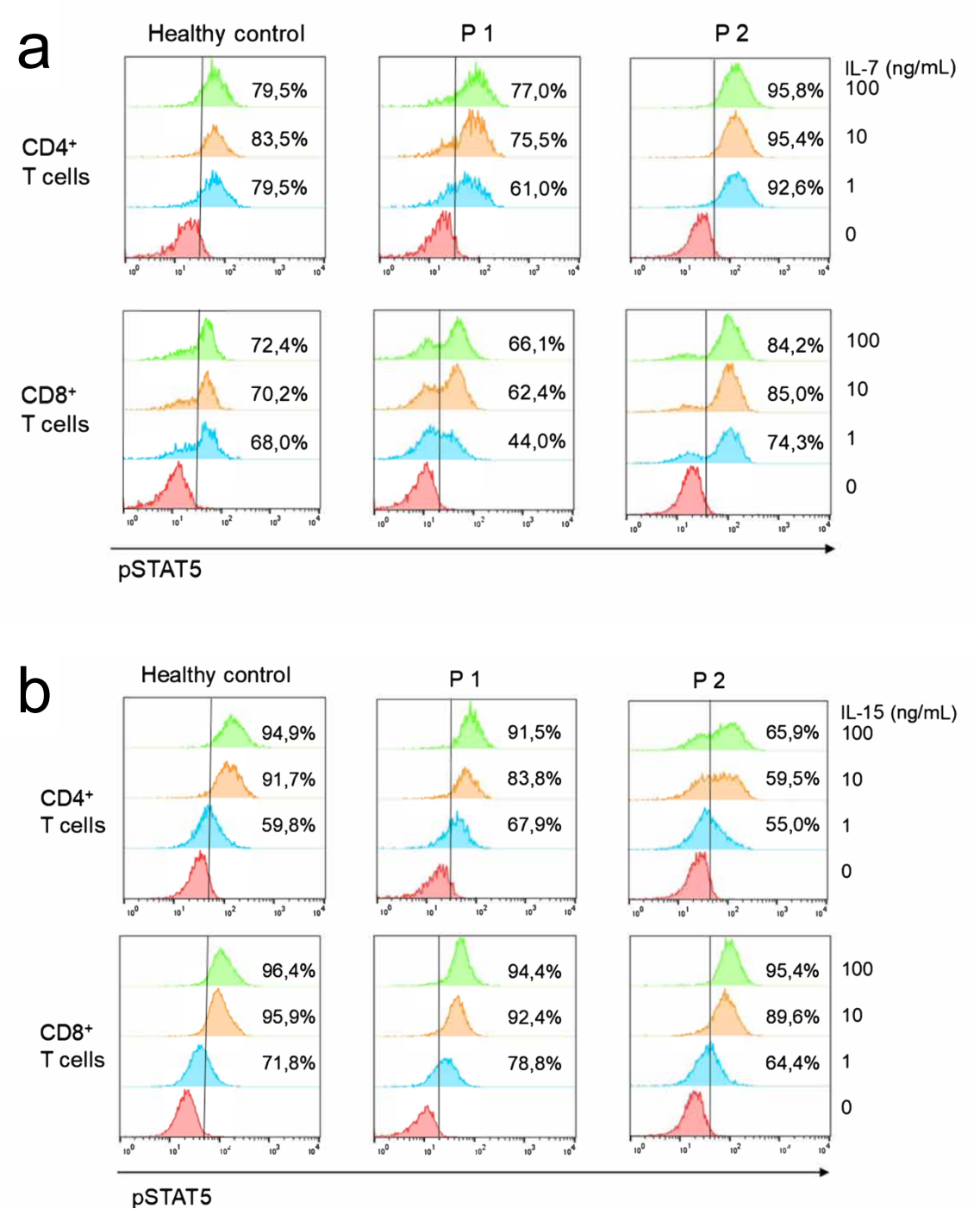



**Figure 7. STAT5 phosphorylation with (a) IL-7 and (b) IL-15 using 1, 10, and 100 ng/mL. A significant dose-depending stimulation was observed for both stimuli. However, no significant difference could be observed in STAT5 phosphorylation from patient cells compared to an age-matched healthy control. [31]**

### *3.3.3 IL2RG mutation analysis in sorted subpopulations*

Mutation of the common gamma chain are usually associated with severe infections and premature death. To further investigate the correlation between the clinical-immunological presentation and genetic finding PBMCs were isolated out of whole blood from all three patients and the cells were sorted. For comparison $CD3^+$ T cells from a healthy donor and the patients' mother were isolated and sequenced serving as a control (**Fig. 8a,b**). The T>C mutation was confirmed by Sanger sequencing in $CD19^+$, $CD56^+$ and $CD14^+$ subsets. Interestingly, $CD3^+$, $CD4^+$ and $CD8^+$ T cells present partial reversion of the mutated base nucleotide revealing a wild-type allele in these cells (**Fig. 8c-e**).

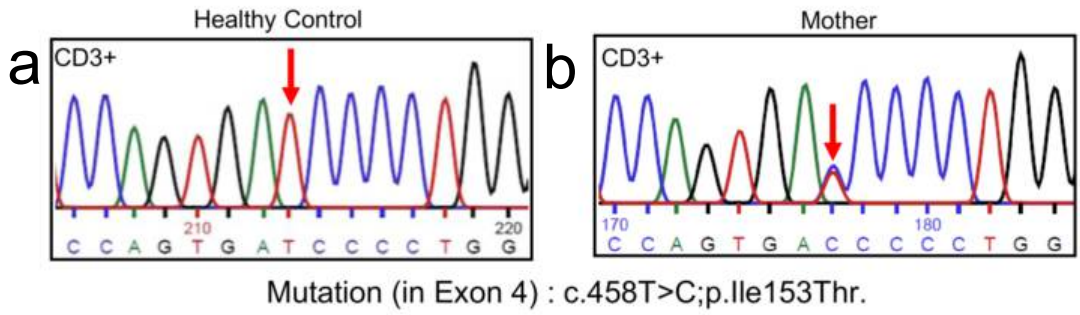



**Fig. 8. Sanger sequencing of $CD3^+$ T cells of (a) healthy control and $CD3^+$ T cells of (b) patients' mother with the same mutation as patients. [31]**

The sequencing results of the healthy donor showed the physiological single-base nucleotide Thymine (T) at position c.458 depicted as a red wave. The patients' mother, however, showed two waves (red and blue) covering each other. This implies for a normal wild-type allele and one single-base nucleotide mutated to Cytosine on each of the X-chromosomes, respectively. Subsequently, the base chance leads to an amino acid substitution from Isoleucine to Threonine at position 153. The difference of the molecular structure of these two amino acids might be responsible for the hypomorphic behavior and will be discussed more detailed later.

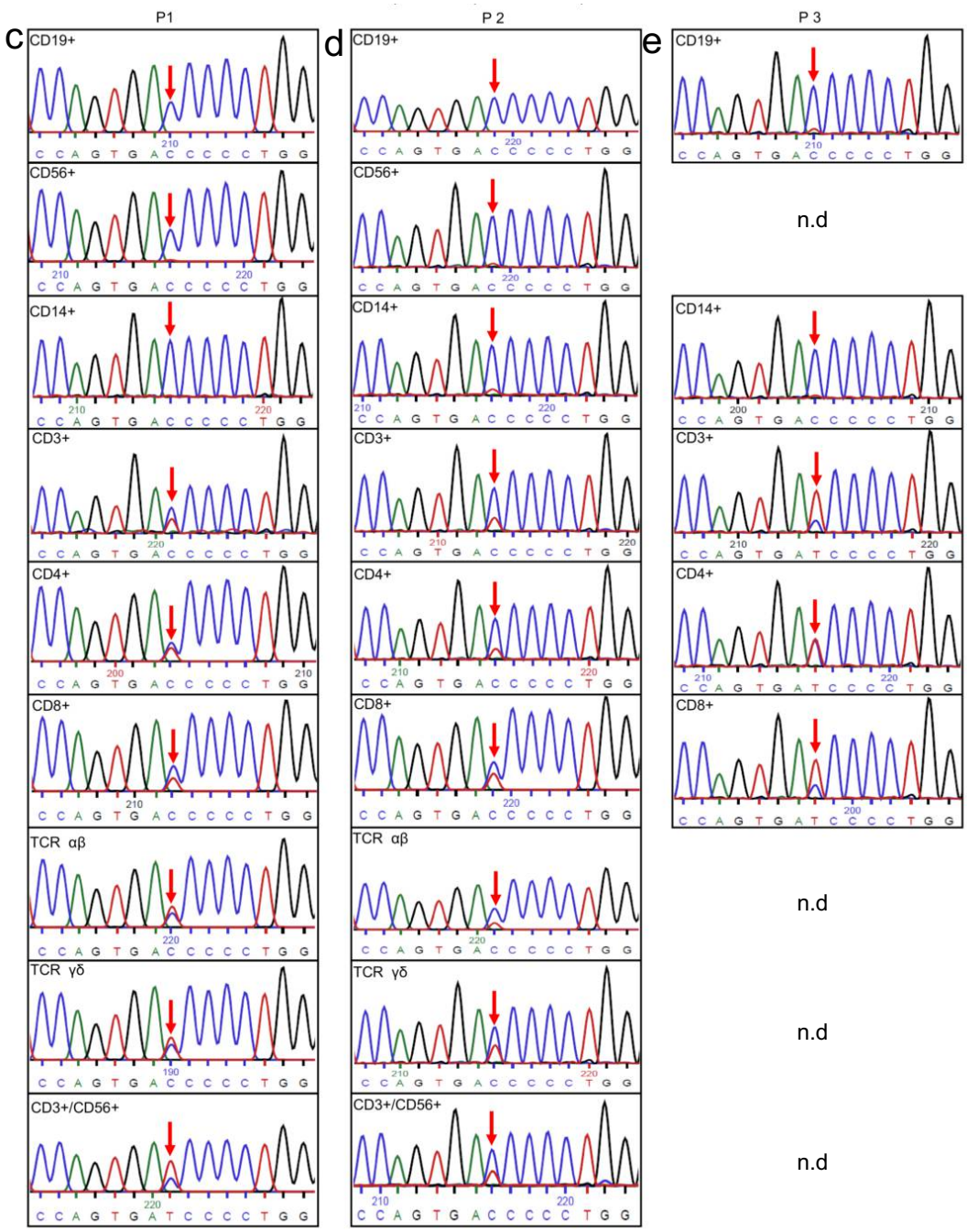



**Figure 8. Various lymphocyte subsets which were sorted from PBMCs using cell sorter of three patients (c, d, e): CD19$^+$ B cells, CD56$^+$ NK cells, CD14$^+$ monocytes showed the mutation, while CD3$^+$, CD4$^+$, CD8$^+$, αβ, γδ T cells and NKT (CD3$^+$/CD56$^+$) cells exhibited certain levels of a revertant mutation. [31] (n.d = not determined due to lack of patient's access).**

### *3.3.4 Maternal engraftment*

To exclude circulating maternal engrafted T cells from the mother to the patients, Short Tandem Repeats (STR) of $CD3^+$ T cells were compared. The results showed dissimilar peaks which proves the relationship while excluding maternal engraftment (**Fig. 9**).

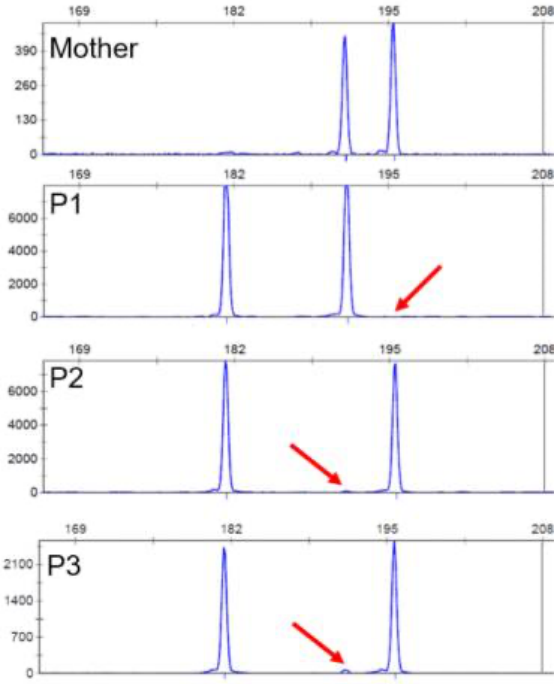


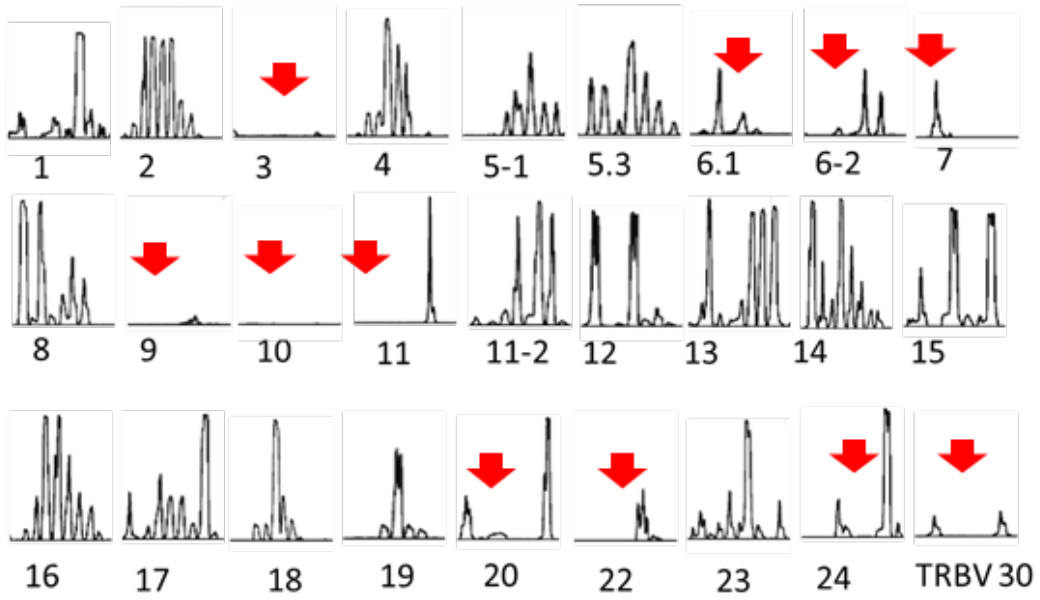

**Figure 9. Typical STR profile with allele designation for genomic DNA extracted from $CD3^+$ T cells of mother and three patients. The horizontal axis represents the values of fragment in base pair and the vertical axis represents values in relative fluorescent units (RFU). [31]**

### *3.3.5 TCR Vβ repertoire analysis*

"The V(D)J rearrangement process generates CDR3 regions varying up to 30 amino acids in length. Amplicons of different lengths show a typical bell-shaped distribution of CDR3 sequences of different lengths with a maximum value of 12-15 amino acids in healthy adults. The CDR3 distribution patterns of all Vβ families of the three siblings with *IL2RG* mutation are dramatically skewed **(Fig. 10)**. The highly significantly reduced TCR repertoire diversity is characterized by a significantly reduced complexity score. The complexity of Vβ- (and Vα-chain)

repertoires can be determined by counting the number of peaks in spectra type analysis. A score of 9 describes a normal CDR3 size variability of 8–10 peaks per Gaussian curve, a score of 1 refers to Vβ family profiles showing single peak, 0 describes the absence of peaks. The overall TCR complexity (complexity score) is the sum of 27 individual TCR Vβ- family scores with a maximum of 27 × 9 = 234 for the Vβ families. P1, P2, P3 showed the score of 64/243 (i.e. 0.26 of normal), 86/243 (i.e. 0.35 of normal), and 78/243 (i.e. 0.32 of normal), separately." [31]

a



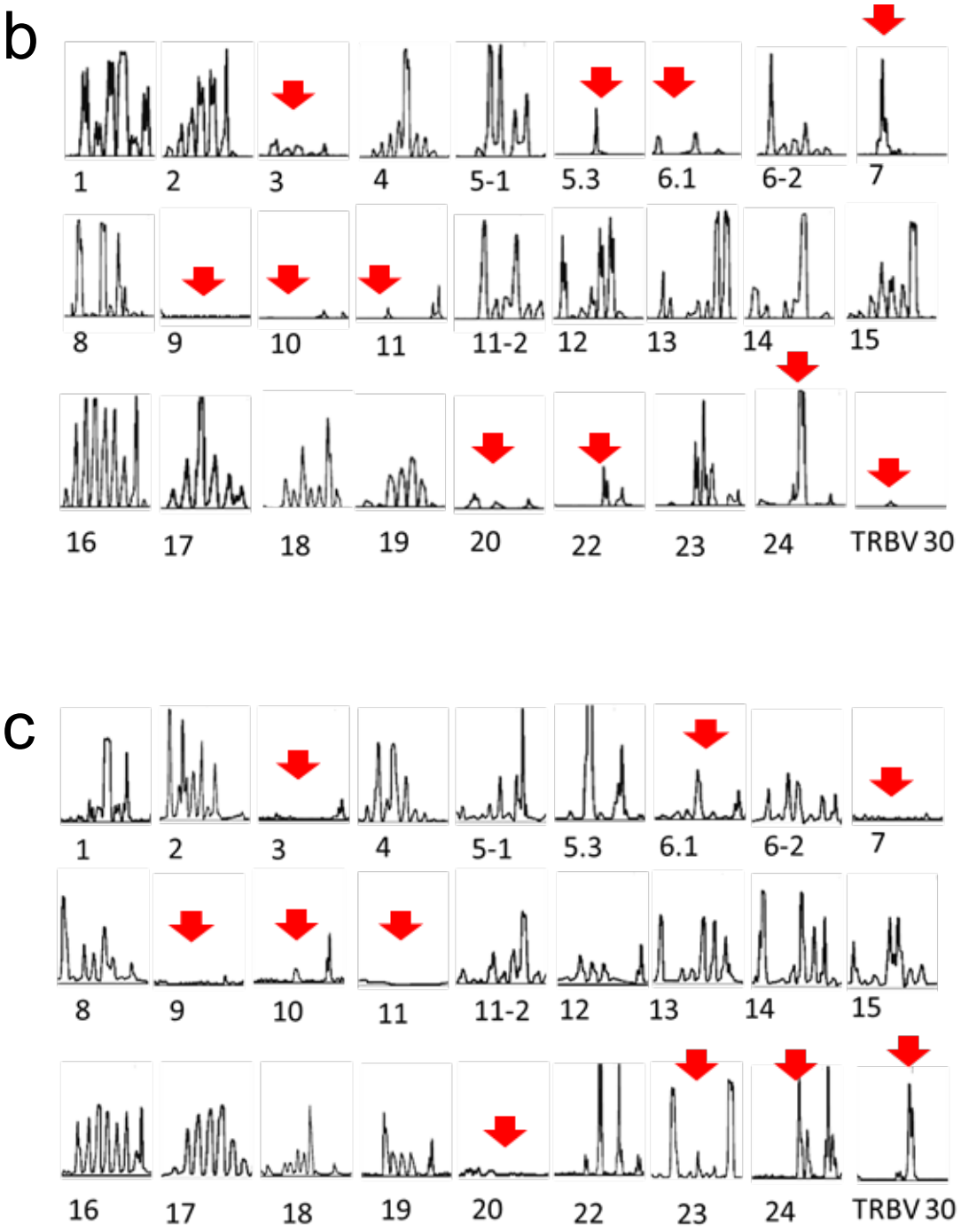



**Figure 10. T-cell receptor repertoire analysis and CDR3 spectratyping: The CDR3 distribution patterns of all Vβ families of the three siblings is significantly skewed. The reduction of TCR repertoire diversity is characterized by a significantly reduced complexity score. TCR Vβ- family scores with a maximum of 27 × 9 = 234 for the Vβ families. (a) P1, (b) P2, (c) P3 showed the score of 64/234 (i.e. 0.26 of normal), 86/234 (i.e. 0.35 of normal), and 78/234 (i.e. 0.32 of normal), separately. [31]**

# 4 Discussion

## 4.1 Hypomorphic mutation leads to partial STAT5 phosphorylation

This study reports three brothers diagnosed with an atypical X-SCID phenotype caused by a novel *IL2RG* missense-mutation (c.458T>C). They all showed cases of recurrent chronic respiratory and viral infections, abnormal immunoglobulins and slightly diminished $CD4^+$ T cells and NK cells. This novel mutation is encoded in the extracellular domain of the *IL2RG* gene and characterized by the substitution of the amino acid Isoleucine at position 153 by Threonine (p.Ile153Thr) and has not been described before. The mild phenotype expression of the patients correlates with the immunological findings of low-normal T and NK cell counts, highlighting the fact that $CD4^+$ and $CD8^+$ T cells only showed partial STAT5 phosphorylation after activation with low concentrations of IL-2 (100 U/mL). Interestingly, the same cells showed an excellent response while being stimulated with high concentrations of IL-2 cytokines (1000 and 10 000 U/mL) or different compounds such as IL-7 and IL-15.

This strengthens the hypothesis of a hypomorphic mutation requiring a specific activation threshold before fully expressing proper IL-2 signaling. Additionally, increasing concentrations of IL-2 showed similar STAT5 phosphorylation as cells of healthy controls if only stimulated sufficiently, which also supports that this novel mutation is hypomorphic [31].

## 4.2 Comparison with other X-SCID cases and possible explanation

Although the p.Ile153Thr mutation is novel and has not been described before, comparable point mutations were detected in the past. Puck et al. [33] previously described a mutation responsible for substituting of Isoleucine with Asparagine at the same position (p.Ile153Asn). In addition, they carried out functional characterization in a following publication which showed that the said mutation does not influence on the expression of the IL-2RG, however, attenuating the binding between IL-2 and its receptor [34]. These results match the characteristics of the novel mutation (p.Ile153Thr) discovered in the three

brothers. The inconsistency between the structural integrity and the impaired function of IL-2RG due to this *IL2RG* mutation requires the deeper study about the function of IL-2RG and immunological of patients. Unfortunately, there was not further report about precise functional analyzation and clinical progression of this patient described by Puck et al. Thus, it can be considered that the patient was classic X-SCID. This different outcome with our cases could be explained in terms of two essential dissimilarities:

- Different effect on protein structure: A closer look at the molecular level revealed that the amino acid Isoleucine at position 153 is part of the extracellular domain of the IL2RG receptor. Here, it plays an important role of proper IL-2 cytokine binding and interaction (**Fig. 11**). Being a hydrophobic amino acid with poor reactive side chains the wild-type isoleucine forms a β-sheet motif, which could be responsible for efficient ligand recognition [35]. On the other hand, asparagine presents with polar side chains and therefore with a more reactive molecular behavior that might not guarantee an undamaged β-sheet motif structure. The replacement of isoleucine with threonine, similar in terms of molecular composition (two non-hydrogen substituents attached to the beta carbon), could preserve the β-sheet structure by limiting the variable formation of the main chain. Thus, it can be hypothesized that the milder phenotype of the p.Ile153Thr mutation is associated with minor consequences in terms of tertiary protein structure [31]. Although it is a rare event (proline in position 154), it is also worth considering that p.Ile153Asn benefits epigenetic modifications in the IL-2RG receptor such as N-linked glycosylations, which additionally would attenuate receptor binding [36].

- Missing somatic reversion in p.Ile153Asn: The identification of this mutation was revealed through a restriction site (*Sau*III*A*) in the mutated cells [34]. However, the existence of somatic reverted mutation would lead to a digestion that is depictable in the gel, which was nowhere mentioned

in the publication. Consequently, to clarify the presence of revertant mutations require further genetical analysis with genomic DNA of all celltypes, especially the in T-, B- and NK cell subsets.

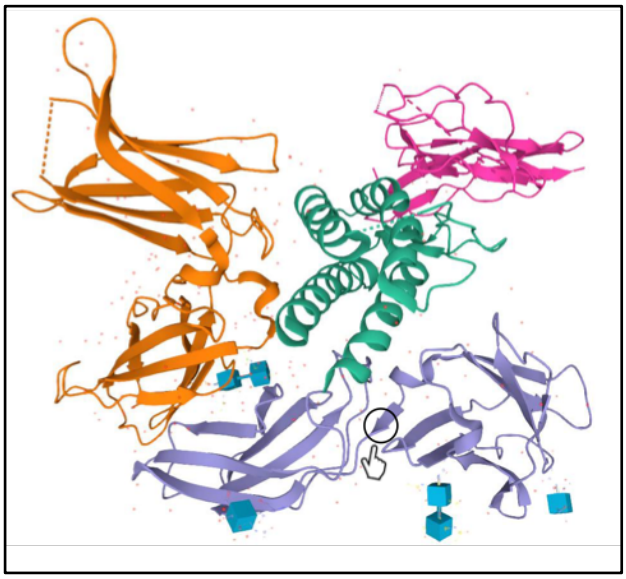


**Figure 11. Molecular structure of IL-2 binding to IL-2 receptor [31]. IL-2 (green), IL2RA (blue), IL-2RB (orange) and IL-2RG (purple). Index points at Isoleucine 153 from IL-2RG. The image was generated with Mol* Viewer application [37] in RCSB PDB (PDB ID: 2B5I [25]).**

### 4.3 Somatic reversion as natural gene therapy in progenitor T cells

Further genetical investigations of lymphocyte subsets displayed somatic mosaicism produced by the revertant mutation in p.Ile153Thr patients. After excluding the possibility of maternal engraftment by STR analysis and the fact that all three siblings carried the same C>T reversion simultaneously it makes it a very unlikely event to happen accidentally. Thus, it can be assumed that a spontaneous somatic reversion process constituted to an unknown genetical mechanism imitating natural gene therapy [31]. Tracing back the developing stages of lymphocytes, it can be presumed that the detected reversion in the subpopulations of the three siblings originated from a revertant progenitor T cell (**Fig. 12**). To consolidate this hypothesis, genetic investigations of TCR αβ+ and TCR γδ+ T cells confirmed that the reversion was detectable in both cell lineages.

One possible stage for the occurrence of somatic reversion is between the $CD4^-$ $CD8^-$ double-negative and $CD4^+CD8^+$ double-positive thymocyte stage, where no CD132 expression is essential for regular T cell development [37].

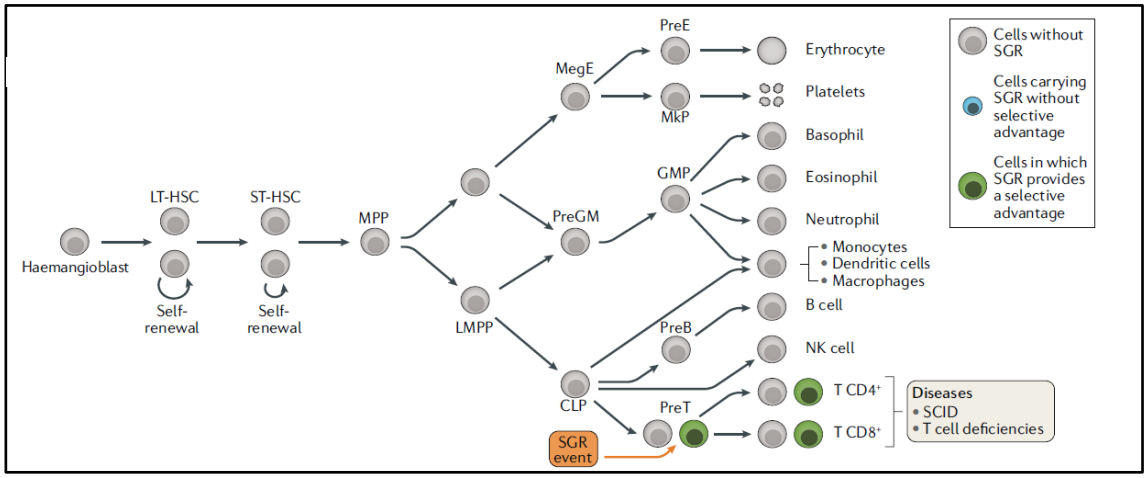



**Figure 12. Example of the somatic reversion process in a single reversal progenitor Pre-T cell and the development of natural corrected T cells (green). Image was adapted from [22].**

Furthermore, it can be hypothesized that the mechanism responsible for the natural gene therapy most likely happened after the lymphoid progenitor cell committed either to B- or NK-cell development because the genetical study did not reveal any somatic reversion in NK-, B cells or monocytes [31]. Another explanation could be that the reversion lead to no significant survival benefit thus NK and B-cells kept the mutated gene type. A broad spectrum of clinical and immunological findings after detection of reverted mutation in the *IL2RG* gene can be observed. For example, low T- and NK-cell numbers were detected in patients with *IL2RG* T452C, T455C and A284-15G mutations [19, 38, 39], normal phosphorylation of STAT5 is documented for a T466C mutation [40], and even normal TCR repertoire in patients with T343C and A655T mutations is known [41, 42]. Therefore, it is crucial to highlight the importance of precise and early functional and immunological analysis to differentiate possible clinical presentations of SCID types especially after the postnatal period.

### 4.4 Low naïve and high memory T cells due to defective IL-2RG signal

The somatic reversion was detected in $CD3^+$ T cells of all three brothers combined with a shifted $CD4^+/CD8^+$ ratio. A similar cellular combination was described by Kuijpers et al. within a 6-year-old boy [43]. They clarified this phenomenon by a smaller proliferation rate in $CD4^+$ T cells compared to the excessive growth and differentiation of $CD8^+$ T cells, which serves as a reasonable explanation for their patient's case. Additionally, P1 presented with decreased amount of $CD3^+CD4^+CD45RA$ naïve T cells and elevated numbers of $CD3^+CD8^+CD28^-CD27^-$ memory T cells (**Table 13**). It is worth highlighting the interesting observation of normal STAT5 phosphorylation in all three patients after stimulation with IL-7 and IL-15 compared to healthy controls (**Fig. 7a,b**). Especially (other than naïve T cells), memory T cells depend much more on IL-7 and IL-15 than on IL-2 in terms of proliferation and differentiation [44]. This molecular behavior through cytokines could explain the survival benefit of memory T cells in comparison with naïve T cells.

**Table 13. Surface marker analysis of peripheral blood mononuclear cells. [31]**

| | P1 (%) | | Reference (%) [45-47] |
|---|---|---|---|
| $CD3^+$ | **85,10** | + | 55,00 – 83,00 |
| $CD4^+$ | **21,90** | - | 28,00 – 57,00 |
| $CD8^+$ | **59,10** | +++ | 10,00 – 39,00 |
| $TCR\alpha\beta^+$ | 96,20 | | 88,00 – 98,00 |
| $TCR\gamma\delta^+$ | 3,70 | | 1,00 – 12,00 |
| $CD3^+CD4^+CD45RA^+$ | **10,00** | -- | 21,00 – 58,00 |
| $CD3^+CD4^+CD45RO^+$ | **85,90** | ++ | 35,00 – 73,00 |
| $CD3^+CD8^+CD45RA^+$ | **4,92** | --- | 23,00 – 73,00 |
| $CD3^+CD8^+CD45RA^-$ | 22,87 | | 13,00 – 43,00 |
| $CD3^+CD8^+CD28^-CD27^-$ | **53,90** | ++ | 1,60 – 36,00 |
| $CD19^+$ | 13,20 | | 6,00 – 19,00 |
| $CD16^+CD56^+$ | **22** | --- | 90 – 600 |

Relative numbers of cells expressing surface markers are shown.

## 4.5 Long-term immunity depending on skewed Vβ TCR repertoire

Interestingly, the three brothers share highly analogical expression of Vβ families especially those not being expressed at all as well as peaks diminished to only one peak. It was previously discussed that these reduced phenotypes might be due to mono or even oligoclonal expansion [48, 49]. Additionally, it can be suggested that virus specific public TCRs immensely contribute to the skewed TCR repertoire. This is in line with the results of the immunological investigation showing more memory T cells compared to naïve T cells (**Table 13**). Consequently, a survival advantage for memory T cells can be concluded out of this assumption (**Fig. 12**). Moreover, the skewed TCR repertoire could be explained by impaired or diminished expression of TCRs, as done previously [50], but due to lack of qualitative samples was not possible in this study. After summarizing the immunological expression of TCR of the T cell compartment of the three brothers it can be presumed that the T cells are functionally impaired, which might explain the mild phenotype. In total, it is crucial to mention that the skewed TCR repertoire in combination with the low TREC levels might lead to T cell depletion with time. Therefore, close follow-up of all three patients is mandatory to clarify if the T cell immunity is maintained in the long-term.

## 4.6 Conclusion

### *4.6.1 Natural somatic reversion as a model for artificial gene therapy*

Overall, it can be concluded that additional factors must contribute to the patients' clinical status, which explains the picture of sharing an identical genetic mutation while expressing a broad variety of symptoms. Possible factors might occur from their epigenetic profile, not to forget external environmental influences, which both undermine the unique development of the brothers' symptomatic conditions, especially highlighting P1 from his two siblings. The close clinical surveillance of P1 showed that his immunological conditions can be restored for at least 26 years. This makes him the longest surviving patient out of eight reported atypical X-SCID cases until now [38, 51-57]. To get deeper insides of the immunological restoration triggered by the natural somatic reversion further studies and analysis

of progenitor T cells are required. However, due to ethical reasons this could not be integrated into this work. Considering the positive outcome of natural somatic reversion and its advantageous impact on revertant cells, it is worth highlighting that novel genetic tools such as CRISPR/Cas9 or prime editing might serve as a promising therapy for the three brothers should their health status worsen with age. [31]

### *4.6.2 Future perspectives and follow-up*

Once more, this work highlights the importance of early genetic and functional testing of patients suspected with SCID despite absence of specific laboratory parameters and missing family history. Early diagnosis is crucial to avoid severe cases of late onset immunodeficiencies. Overall, the remaining question whether the partial STAT5 phosphorylation and the T-cell repertoire of the revertant progenitor cells provides the patients with sufficient long-term immunologic protection cannot be answered based on current information. Therefore, frequent and close follow-up of the patients is urgently recommended and already administrated by the outstanding care of the University Children's Hospital, Tübingen. The evaluation of the patients' functionality and longevity of the life-saving somatic reversion will play a major role of careful surveillance.

**Table 14. Characteristics of reported patients carrying the IL2RG c.458T>C; p.Ile153Thr hypomorphic mutation. [31]**

| | P1 | P2 | P3 |
|---|---|---|---|
| Thymic shadows | n.a. | n.a. | n.a. |
| Blood count | CD4+ and NK cell lymphopenia | Low CD4+ T cells | Low CD4+ T cells |
| Immunophenotype | $T^{low}B^{+}NK^{low}$ | $T^{low}B^{+}NK^{low}$ | $T^{low}B^{+}NK^{low}$ |
| γδT cells | Normal | Normal | Normal |
| Extended immunophenotype | Low CD4/CD8 ratio <1.0 | Low CD4/CD8 ratio <1.0 | Low CD4/CD8 ratio <1.0 |
| Immunoglobulin levels | Dysgammaglobulinemia | Dysgammaglobulinemia | Dysgammaglobulinemia |
| Lymphocyte proliferation | Variable | Variable | Variable |
| TRECs | Reduced | Reduced | Reduced |
| TCR Vβ repertoire | Skewed | Skewed | Skewed |
| Genetic findings | *IL2RG* c.458T>C; p.Ile153Thr | *IL2RG* c.458T>C; p.Ile153Thr | *IL2RG* c.458T>C; p.Ile153Thr |
| IL-2RG expression | Normal | Normal | Normal |
| STAT-5 Phosphorylation | Partially defective (FC) | Partially defective (FC) | n.a. |

FC = flow cytometry; NK = natural killer; TCR = T cell receptor; TRECs = T cell receptor excision circles; n.a. = not assessed.

# 5 Summary

The interleukin-2 receptor common gamma chain gene (IL2RG) encodes for the common gamma chain ($y_c$), which is a part of multiple interleukin receptors such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptor. T cell proliferation and cytokine production is induced due to its activation of Janus kinase 3 (JAK3) and signal transducer and activator of transcription 5 (STAT5) depended signaling pathway. Therefore, mutations in this gene can cause impaired cell-mediated and humoral immunity leading to severe combined immunodeficiency (SCID). From birth patients suffer from total absent or greatly reduced T and NK cells being susceptible to opportunistic pathogens, which shows in recurrent respiratory and viral infections. Usually, the infants die within the first year of life unless undergoing prompt hematopoietic stem cell transplantation or gene therapy. Lately, hypomorphic mutations in the IL2RG gene with milder phenotypes have been described. Unlike SCID, the cells of the lymphatic lineage can be detected in peripheral blood count even if they display functional impairment. This work focuses on the functional characterization of a novel c.458T>C; p.Ile153Thr IL2RG missense-mutation observed in three brothers. They were diagnosed with atypical SCID due to their milder phenotype expression. Functional characterization of the common gamma chain (γc) revealed its hypomorphic function. In addition, we detected somatic reversion predominantly in lymphoid derived subpopulations. The combination of hypomorphic IL2RG function and somatic reversion explains the atypical phenotype of the patients and serves as a model for novel gene editing tools (e.g., CRISPR/Cas9) imitating the process of natural gene therapy.

# 6 Zusammenfassung

Das IL2RG Gen kodiert für die common gamma Kette ($y_c$), welche Teil von vielen Interleukin Rezeptoren wie IL-2, IL-4, IL-7, IL-9, IL-15 und IL-21 Rezeptor ist. Durch Ihre Aktivierung kommt es über eine Janus Kinase 3 (JAK3) und den Signal Transducer and Actviator of Transcription 5 (STAT5) zu einer Proliferation von T-Zellen und der Produktion von Zytokinen. Mutationen dieses Gens führen daher zu zellulären und/oder humoralen Einschränkungen des Immunsystems bis hin zu schweren kombinierten Immundefekten (SCID). Betroffene Patienten leiden unter totaler Abwesenheit oder starker Verminderung der T- und NK-Zell Zahl, was sie für opportunistische Erreger äußerst anfällig macht. Wiederkehrende respiratorische und virale Infektionen treten daher gehäuft auf und können schwere Verläufe mit sich bringen. Ohne rechtzeitige Hämatologische Stammzelltransplantation bzw. Gen Therapie versterben die betroffenen Säuglinge gewöhnlich innerhalb des ersten Lebensjahrs. Zuletzt wurden aber auch hypomorphe Mutationen mit milderen Symptomen beschrieben. Zwar konnten hier lymphatische Zellen im peripheren Blut nachgewiesen werden, allerdings mit funktionellen Einschränkungen. Diese Arbeit handelt von der funktionellen Charakterisierung einer neuartigen c.458T>C; p.Ile153Thr IL2RG Missense Mutation, welche in drei Brüder beobachtet wurde. Aufgrund der milden Phänotyp Expression wurde die Diagnose „atypical“ SCID gestellt. Funktionelle Untersuchungen ergaben die für eine hypomorphe Mutation charakteristischen funktionellen Einschränkungen. Zudem stellte sich interessanterweise heraus, dass einige lymphatische Zellen eine gesunde Wildtyp Allelfrequenz aufwiesen. Diese Kombination aus hypomorpher IL2RG Funktion und somatischer Reversion liefert die Erklärung für den milden Verlauf des sonst mit schweren Verläufen assoziierten Immundefekts. Zukünftig könnte dieser molekulare Mechanismus als Vorlage für neue Gen Therapien wie z.B. CRISPR/Cas9 dienen.

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## 8 Statement

I hereby declare

- that this thesis is my own work and that I have not made use of any other sources or aids than those referenced.
- that all statements appropriated from other works in letter or in substance have been referenced as such.
- that the copy submitted in file format corresponds to the bound copy submitted.

## 9 Publications

Parts of this thesis have already been published in the following publications:

**Yujuan Hou†, Hans Peter Gratz†, Guillermo Ureña-Bailén, Paul G Gratz, Karin Schilbach-Stückle, Tina Renno, Derya Güngör, Daniel A Mader, Elke Malenke, Justin S Antony, Rupert Handgretinger, Markus Mezger - Somatic Reversion of a novel *IL2RG* Mutation resulting in Atypical X-linked Combined Immunodeficiency - Genes - 2022**

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