AMERICAN UNIVERSITY OF BEIRUT

SEVERE COMBINED IMMUNODEFICIENCY DUE TO A NOVEL MISSENSE MUTATION IN IL-7Rα THAT ABOLISHES SURFACE PROTEIN EXPRESSION

by
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ABSTRACT
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Title: Severe Combined Immunodeficiency Due to a Novel Missense Mutation in IL-7Rα that Abolishes Surface Protein Expression

Background: Primary immunodeficiency diseases (PIDs) are a group of congenitally inherited heterogeneous disorders that result in depletion or deficiency of one or more components of the immune system. Severe Combined Immunodeficiency Diseases (SCID) are the most severe and life-threatening subgroup of PID as they affect the development and/or function of T cells, predisposing the patients to recurrent infections and autoimmune manifestations at an early age. IL-7R, the cytokine receptor for interleukin- (IL-) 7, is expressed on lymphoid cells and plays an important role in the proliferation, development, homeostasis, and survival of T cells. Defects in IL-7R abolish T cells resulting in $T^-B^+NK^+$ SCID.

Case Presentation: The patient under investigation is a 1 year-old female born to consanguineous parents. Her clinical history is indicative of decreased T cell counts associated with recurrent severe infections and autoimmune hemolytic anemia since birth. The early onset and severity of the clinical manifestations were suggestive of SCID.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-paque gradient by differential centrifugation. Immunophenotyping of PBMCs, expression of the IL-7Rα, Regulatory T cell staining, and T cell proliferation were performed by flow cytometry. Genomic DNA (gDNA) was purified and sequenced by Next Generation Sequencing and Sanger sequencing. RNA was extracted using TRI reagent, reverse transcribed to cDNA using RNA reverse transcription kit, and amplified by PCR followed by Sanger sequencing.

Results and discussion: Immunophenotyping of PBMCs revealed a very low T cell count, absent Recent Thymic Immigrants (RTEs) and B cells, and normal to elevated numbers of NK cells. Importantly, the few patient T cells failed to proliferate when stimulated with anti-CD3 monoclonal antibodies (mAb) or Phytohaemagglutinin (PHA). Next Generation Sequencing analysis identified a homozygous missense mutation in *IL7Rα* gene (c.379 G>A) that resulted in a Valine to Isoleucine change at position 127 of the protein (p.V127I). The c.379G>A mutation resulted in aberrant RNA splicing and abolished cell surface IL-7Rα protein expression, as determined by

flow cytometry. These results demonstrate that the patient harbors a deleterious mutation in IL-7Rα resulting in a $T^-B^+NK^+$ phenotype, thus confirming the clinical diagnosis of SCID.
The patient was successfully transplanted at the AUB-MC using her HLA-matched relative as donor. Ten months after transplant, the patient showed significant increase in the percentage of lymphocytes and T cells, reconstitution of T cells that express IL-7Rα, and restoration of T cell's proliferative response to mitogens.

Conclusion:
Using genetic and functional approaches, we identified a SCID patient with a novel deleterious homozygous mutation in the *IL7Rα* gene and studied the effect of the mutation on protein expression and T cell function. The patient was transplanted at AUBMC and we were able to follow the reconstitution and function of donor cells with time. To our knowledge, this represents the first bedside-to-bench and back to bedside project entirely executed on a patient with SCID at AUB and paves the way for more similar studies in the future.

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CHAPTER I

INTRODUCTION

The immune system is composed of an innate and an adaptive component that work in perfect synchrony to provide protection against foreign antigens (Ags), which either cause disease (pathogen) or an exaggerated non-infectious response (allergen)[1]. The innate immune system is the first line of defense responsible for acting rapidly against Ags, whereas the adaptive immune system responds relatively later as it goes through a process of specific and detailed examination of the Ag before mounting a response against it[2].

Deficiencies of one or more components of the immune system could occur, and are divided into primary and secondary immunodeficiencies[3]. Secondary immunodeficiencies are acquired as a result of malnutrition, viral infections, chemotherapy, irradiation, the use of immunosuppressive drugs, biological drugs, and anti-inflammatory drugs, as well as secondary to metabolic disorders[4].

On the other hand, primary immunodeficiencies (PIDs), also referred to as Inborn Errors of Immunity (IEI), are a group of genetic disorders that result in depletion or functional deficiency of one or more components of the immune system[5,6.] PIDs are very heterogeneous in their manifestations resulting in various combinations of recurrent infections, allergies, dermatitis, enteritis (diarrhea, vomiting, malabsorption), and increased risks of autoimmunity and malignancies[3,6]. The clinical manifestations of PIDs range from mild, as in the case of selective IgA deficiency (SIGAD), to severe and sometimes life-threatening, as in the case of Severe Combined Immunodeficiency Diseases (SCID)[7,8]. SCID is caused by failure of normal T cell function due to

development, differentiation, survival, or proliferation defects that can also be associated with B and/or NK cell defects depending on the gene involved[7].

PIDs are most prevalent in the registries of Middle East and North Africa (MENA) regions relative to other regions where consanguineous marriages are common with an incidence of 20-50%, which favors the segregation and affluence of disease-causing mutations specifically AR (autosomal recessive) disorders in the population[9,10]. Studies have shown that 60% of the genetic disorders that have been recorded in Arabs are found to be AR According to statistics; 30% of Tunisian, 44% of Oman, 48% of Iranian, 61% of Saudi Arabian, and 80% of Egyptian patients have a recorded family history of PID[9]. PIDs have been historically underdiagnosed because of their rare occurrence and the challenge of their diagnosis[11,12]. Conventional treatments for PIDs include the use of antibiotics, steroids, and intravenous immunoglobulin (IVIG)[6]. However, these approaches are supportive temporary treatments because they handle the symptoms but do not cure the underlying disorder[6]. The curative treatments of PIDs include enzyme replacement, hematopoietic stem cell transplantation (HSCT), and gene therapy[6]. In the absence of a clear clinical and definite genetic diagnosis, these curative treatments are usually not provided, resulting in increased morbidity, a shorter life expectancy, and an increased burden on the patients, families, and healthcare system[12].

In 2018, Lebanon introduced a national newborn screening program for SCID where every newborn is screened 24-48 hours after birth for the presence of T cells to identify possible SCIDs[10]. In the same year, next generation Sequencing (NGS) for PIDs was introduced in the Immunology Research Laboratory at the Faculty of Medicine of the American University for Beirut. These represented big steps towards early diagnosis of PID in Lebanon, which is not only important for the clinical

treatment of the patients, but also to understand the consequences of genetic variation on the function of the immune system and the development of the disease[12].

In my research work, we identified a patient with SCID due to a mutation in the interleukin-7 receptor alpha chain (IL-7Rα) and determined the effect of the mutation on gene function, RNA and protein expression, and T cell development and function. The patient underwent HSCT, therefore, we also studied the success of donor cell engraftment with time.

CHAPTER II

LITERATURE REVIEW

A. Primary Immunodeficiency Disorders

PIDs are classified into ten groups based on the report from the International Union of Immunological Societies Expert Committee (IUIS)[5]:

1. Combined immunodeficiency
2. Well-defined syndromes with immunodeficiency
3. Predominantly antibody defects
4. Defects of immune dysregulation
5. Congenital defects of phagocyte number, function, or both
6. Defects of intrinsic and innate immunity
7. Autoinflammatory disorders
8. Complement deficiencies
9. Bone marrow failure
10. Phenocopies of inborn errors of immunity

Combined immunodeficiencies (CIDs) are characterized by a range of T cell defects in development and/or function, sometimes accompanied by partial B cell and/or NK cell deficiency[7,13]. The most severe form of CID is known as Severe Combined Immunodeficiency (SCID), and is characterized by complete absence of T cells at birth, or by the presence of dysfunctional peripheral T cells that undergo rapid apoptosis[7,14] .

SCID disorders can be classified based on the T, B, and NK cell phenotype into $T^-B^+NK^+$, $T^-B^-NK^+$,$T^-B^+NK^-$, and $T^-B^-NK^-$[7]. Patients with SCID are predisposed to recurrent viral, bacterial, and fungal infections sometimes associated with syndromic

features such as growth failure, diarrhea, and eczema or rashes[14]. The only curative treatments for SCID are enzyme replacement, HSCT, and gene therapy[14].

SCID can also be sub-divided into six separate categories based on the pathogenic mechanism resulting in the disorder[15]:

1. Defective survival of hematopoietic precursors
2. Toxic metabolite accumulation
3. V(D)J recombination abnormalities
4. TCR abnormalities
5. Thymic Abnormalities
6. Cytokine signaling anomalies

Figure 1 summarizes the developmental stages of immune cells and shows the different stages at which a blockage can result in immune cell's maturation defects: 1) Defective survival of hematopoietic precursors due to Reticular Dysgenesis (AK2 Deficiency), 2) Toxic metabolite accumulation due to ADA and PNP deficiency leading to lymphocyte precursor death, 3) Cytokine signaling anomalies that include IL-2Rγ and JAK3 deficiency that abrupt the development of T and NK cells, and IL-7Rα deficiency that selectively inhibits T cell development, 4) V(D)J recombination abnormalities due to deficiency in RAG1, RAG2, Artemis, DNA Ligase IV, XLF, and PKCs that result in defective T cell receptor (TCR) and B cell receptor (BCR) rearrangement, 5) TCR functional abnormalities due to mutations in genes responsible for TCR expression and signaling such as CD45, CORO1A, CD3ε, CD3δ, and ζ chain[16,17].

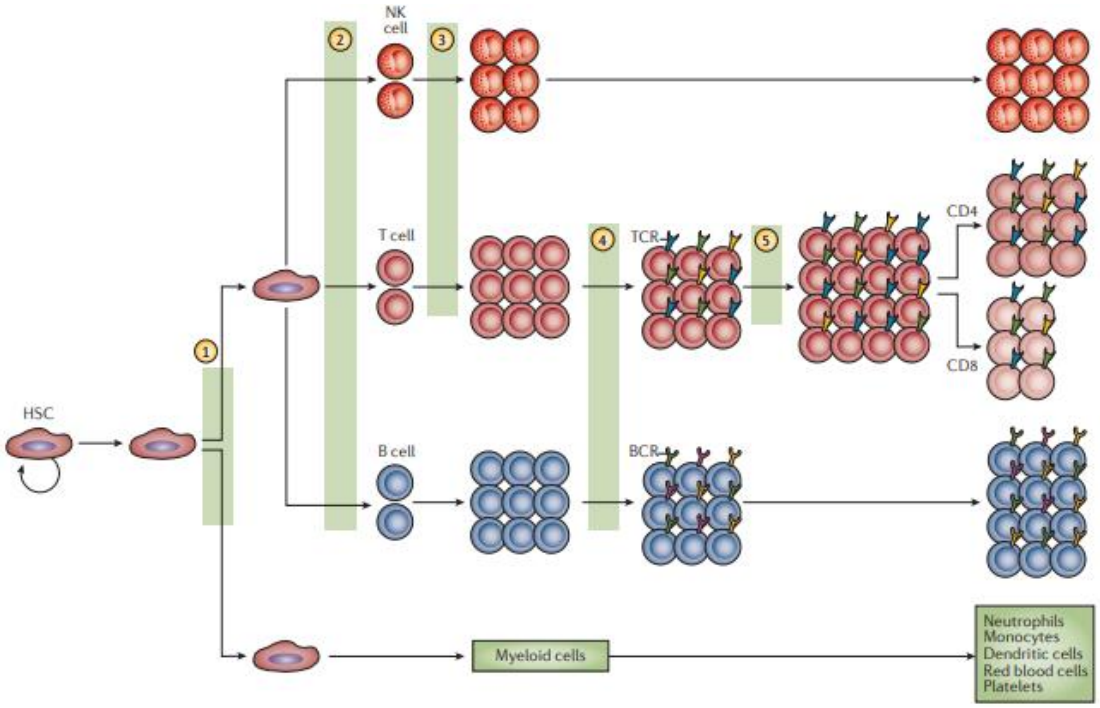

Figure 1.Schematic representation of immune cell development stages with focus on defects in lymphoid cell development that result in SCID.

1. Defective survival of hematopoietic precursors

Reticular Dysgenesis (RD) is an autosomally-inherited disorder that is a very severe form of SCID resulting in a $T^-B^-NK^-$ phenotype[15]. Patients with RD have a failure in the differentiation of myeloid and lymphoid lineages (Figure 1), and are thus depleted of any lymphocytes and granulocytes in the peripheral blood[7]. The defect lies in a mutation of the *Adenylate kinase 2* (*AK2*) gene that encodes the AK2 protein found in the intermembrane space of the mitochondria[15,18]. The function of this protein is to regulate the transport of the nucleotide adenine, one of the building blocks of DNA and RNA, inside the mitochondrial space, and is thus important for mitochondrial function[18]. Failure of AK2 function leads to clinical manifestations including sensorineural deafness and severe recurrent infections[7]. The method of treatment for

AK2 deficient patients is through HSCT which replaces their bone marrow with stem cells from a healthy donor that express wild type AK2[15].

2. Toxic Metabolite Accumulation

Adenosine Deaminase (ADA) and Purine Nucleoside Phosphorylase (PNP) are enzymes that play a significant role in the purine salvage pathway (Figure 2)[15].

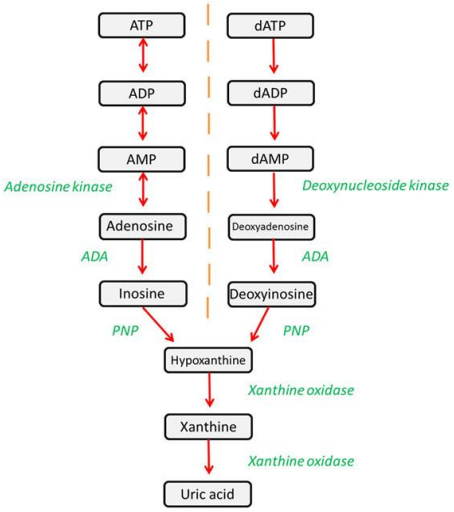

Figure 2. Role of ADA and PNP enzymes in the Purine Salvage Pathway[19].

A Deficiency in any of these enzymes results in an autosomal recessive form of $T^-B^-NK^-$ SCID[15]. ADA is an enzyme responsible of deaminating adenosine and deoxyadenosisne to inosine and deoxyinosine[15]. ADA's function is essential in actively proliferating cells such as lymphocytes[20]. Therefore, a deficiency in ADA results in the accumulation of adenosine and deoxyadenosine metabolites that are toxic to the cells[20]. As a result, lymphoid precursors undergo apoptosis in the bone marrow and thymus and patients suffer from bone marrow and thymic hypoplasia and absence of lymphocytes in the periphery[7]. Clinical manifestations of this disorder include: deafness, behavioral

problems, costochondral abnormalities, liver toxicity, progressive neurological deterioration, autoimmune hemolytic anemia (AIHA), and recurrent opportunistic infections[7].

PNP is an enzyme that converts inosine and deoxyinosine to their respective purine base hypoxanthine by a process known as phosphorylisis (Figure 2)[15]. PNP deficiency affects mainly T cells due to the accumulation of deoxyguanisone that results in toxicity and DNA damage (Figure 2)[15]. Patients with PNP deficiency usually exhibit lymphopenia along with hypouricemia, a biological marker that can be used for the identification of PNP deficiency disorder[15]. Clinical manifestations also include ataxia, developmental delay, spasticity, hemolytic anemia, sclerosing cholangitis, megaloblastic anemia, and sometimes a dysplastic bone marrow[15]. Treatment of ADA and PNP deficiency is through enzyme therapy for ADA only, HSCT, or gene therapy[15].

3. V(D)J Recombination abnormalities

V(D)J recombination abnormalities in RAG1/RAG2, Artemis, PKcs, DNA ligase IV, and XLF/Cernunnos are autosomal recessive inherited disorders that result in $T^-B^-NK^+$ SCID[15]. These proteins play a crucial role in the process of V(D)J recombination that starts with the 1) formation of a DNA loop known as synapsis, followed by the 2) formation of double stranded DNA breaks mediated by a heterodimer of RAG1 and RAG2, 3) hairpin opening and end processing conducted by Artemis and PKcs, then 4) linking of the DNA breaks known as non-homologous end joining mediated by XLF and DNA ligase IV[21] (Figure 3). This process results in the formation of functional TCR and BCR[21]. Therefore, a deficiency in any of these genes

results in arrest of T and B cell development, due to the failure of V(D)J rearrangements that is crucial to form the TCR and BCR[7] .

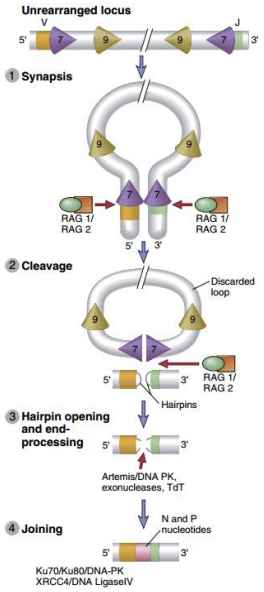

Figure 3. V(D)J recombination process with the corresponding enzymes involved in the recombination steps[21].

4. TCR abnormalities

CD45 is a protein tyrosine phosphatase that plays an essential role in the signaling pathway downstream of the TCR, and is crucial for the T cell development in the thymus[15]. CD45 deficiency is an autosomal recessive disorder that results in $T^-B^+NK^+$ SCID[15]. In addition to that, CD45 is also involved in B cell maturation[15], therefore, patients with CD45 deficiency have a low number of T cells and nonfunctional B cells[15].

CD3ε, CD3δ, and the ζ chain form part of the TCR multimeric complex[15], are the signaling components of the TCR, and are thus important for T cell development[15,7]. Deficiency of CD3ε, CD3δ, and the ζ chain results in an autosomal recessive $T^-B^+NK^+$

SCID with high incidence of recurrent infections. It is also associated with autoimmunity such as AIHA, vitiligo, Hashimoto's thyroiditis, autoimmune enteropathy, Evans syndrome, auto-immunehepatitis, and nephrotic syndrome[15].

CORO1A functions as an enzyme that regulates the activity and assembly of F-actin filaments[22]. It is needed by T cells to egress out of the thymus, thus a deficiency in this enzyme results in T cell retention in the thymus[15,22]. CORO1A deficiency is an autosomal recessive disorder characterized by $T^-B^+NK^+$ SCID with absent peripheral T cells[15]. Patients with CORO1A deficiency are susceptible to recurrent infections[15].

5. Thymic Abnormalities

FOXN1 is an essential transcription factor found in several epithelial cells of the thymus, hair, nail, and skin[23,24]. It is needed for the proper development and differentiation of thymic epithelial cells[23,24]. Deficiency in FOXN1 causes a rare autosomal recessive form of Nude/SCID with $T^{-/low}B^+NK^+$ phenotype[23]. Because of an absent or abnormally developed thymus, T cell development is affected and the patients have low number of T cells in the periphery[23]. Clinical manifestations of this disease include susceptibility to infections associated with thymic hypoplasia, nail dystrophy, and alopecia universalis[23,25].

DiGeorge Syndrome is an autosomal dominant disorder or *de novo* mutation that results in $T^-B^+NK^+$ SCID[15]. It is also known as 22q11.2 deletion syndrome and it varies in the size of deletion that takes place at the long arm of chromosome 22[26]. This deletion impairs thymus and parathyroid glands development, leading to thymic hypoplasia, hypoparathyroidism, conotruncal defects of the heart, cognitive defect, birth defect, facial disfiguration, feeding difficulties, gastrointestinal disorders, and increased

frequency of psychiatric disorders[15,26]. As a result of defective thymic development, the numbers of peripheral T cells are low to absent depending on the size of the deleted 22q11.2 DNA fragment and the genes involved in the deletion[15].

6. Cytokine Signaling Anomalies

Mutations in IL-2Rγ result in an X-linked form of SCID that is the most common form in males, characterized by $T^-B^+NK^-$[7]. IL-2Rγ is also known as the common γchain (γc) because it is shared by several cytokine receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21[16,27]. Since IL-7 and IL-15 are needed for the development and function of T and NK cells, mutation in the γc chain results in depletion of T and NK cells, and increased susceptibility to recurrent infections[7,15] . Treatment for γc deficiency includes HSCT and gene therapy[28].

JAK3 is an essential tyrosine kinase that interacts directly with the γc chain[15]. Upon binding of the cytokine to the corresponding receptor, JAK3 transduces the signals downstream of the γc receptor leading to autophosphorylation, as well as phosphorylation and activation of STAT proteins that translocate to the nucleus and initiate the transcription of genes important for T cell function[15,29]. Mutations in JAK3 cause autosomal recessive $T^-B^+NK^-$ SCID with a clinical phenotype identical to that of patients with γc deficiency[7]. Treatment for JAK3 deficiency includes HSCT[30].

a. <u>IL-7R</u>

Interleukin-7 Receptor (IL-7R) will be elaborately described because it is the main subject of my thesis work. IL-7R is the cytokine receptor for interleukin- (IL-) 7[31]. It is expressed on the surface of lymphoid cells and plays an important role in the

proliferation, development, homeostasis, and survival of T cells at all developmental stages (pro-T, pre-T and mature T cells) in the thymus, and to regulate the selection of self-tolerant T cells[31,27]. IL-7R is also important for B cells to complete their maturation in the bone marrow[31].

Common lymphoid progenitors (CLP) in the bone marrow give rise to T cell precursors (pro-T cells) that migrate to the thymus where they complete their maturation[29]. The maturation of T cells goes through three defined stages[29]. Double negative ($CD4^-CD8^-$) T cells progress through the double positive ($CD4^+CD8^+$) stage and differentiate into single positive ($CD4^+$ or $CD8^+$) mature T cells[29]. The IL-7R is expressed in the double negative stage (pro- and early pre-T cells), silenced in the double positive stage, and re-expressed in the single positive stage[29]. In the double negative stage, the purpose of IL-7R expression is to maintain the survival of the T cells and help with the rearrangement of the TCR[29]. IL-7R is then silenced in the double positive stage to prevent the survival of self-reactive T cells that are destined for negative selection in the thymus, in order to limit the development of autoimmune diseases[29]. In the single positive stage, IL-7R is re-expressed to enhance the proliferation of the positively selected T cells, produce survival signals, and potentiate the differentiation into $CD8^+$ T cells[29].

B cell lymphopoiesis takes place in the bone marrow where the CLP goes through several differentiation stages[32]. The IL-7R is expressed through the Pre-Pro B cell until early pre-B cell stage to facilitate the commitment of precursor stem cells to the B cell lineage, the recombination of the V(D)J segments of the BCR, and the survival, maturation, and proliferation of B cells[32].

The IL-7R is a heterodimer of the IL-7Rα chain, a type I transmembrane protein also known as CD127, associated with the γc chain and expressed on the surface of the cells[33]. The IL-7Rα can also complex with the Thymic Stromal Lymphopoietin (TSLP) receptor to form the TSLP-R that binds to the thymic stromal lymphopoietin ligand (Figure 4)[33].

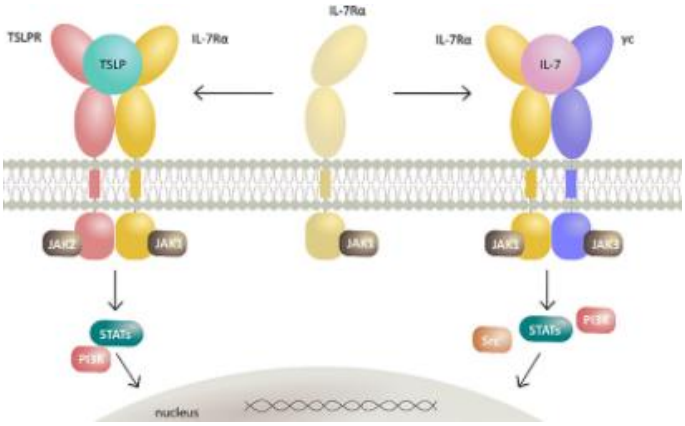

Figure 4. Schematic presentation of the IL-7Rα chain forming a heterodimeric complex with the γc chain and TSLP receptor [34].

IL-7Rα consists of an extracellular domain that, in complex with the γc, binds to IL-7, a transmembrane domain that guides and embeds the protein on the surface of the cells, and an intracellular domain that relays the signal produced by IL-7 to activate downstream signaling molecules (Figure 5)[35].

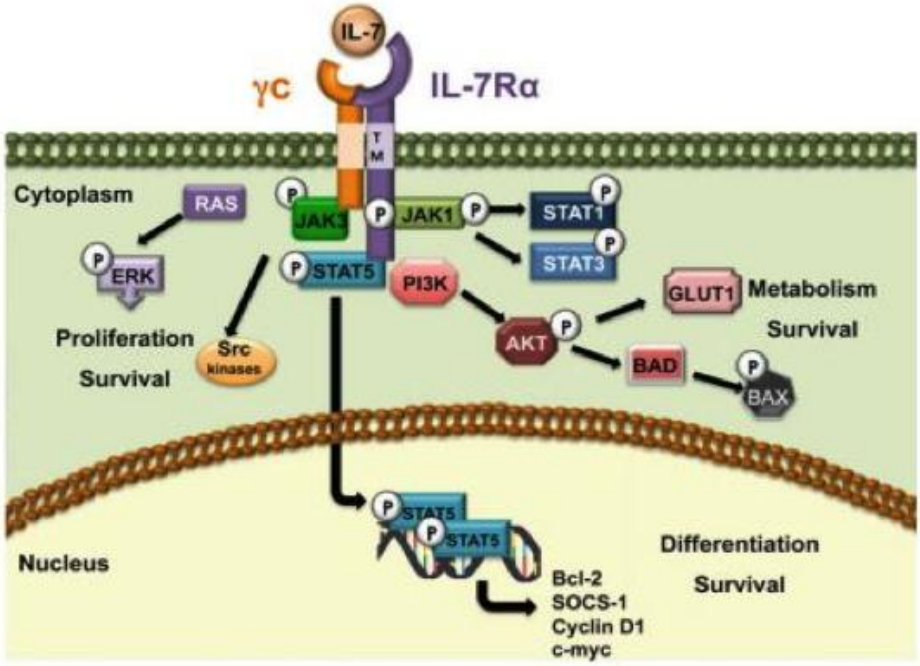

Figure 5. Signaling events downstream of the IL-7R[36].

Binding of IL-7 to IL-7Rα results in its heterodimerization with the γc[36,37]. Then, JAK1 bound to the IL-7Rα tail and JAK3 bound to the γc chain tail get activated and phosphorylate one another to optimize their function[29]. In addition, JAK1 phosphorylates the cytoplasmic tail of IL-7Rα on Y449 providing a docking site for SH2 domain-containing signaling molecules such as Signal Transducers and Activators of Transcription 1, 3, and 5 (STAT 1, STAT3 and STAT 5) and Phospho-inositol 3 kinases (PI3K)(Figure 5)[36,38]. After recruitment to the cytoplasmic tail of the IL-7Rα, STAT5 gets phosphorylated by JAK1, homodimerizes and translocates to the nucleus where it initiates the transcription of *Bcl-2, SOCS-1, CyclinD1 and c-myc*, which play a significant role in the differentiation, proliferation, and survival of the cells[29,31,36]. PI3K is also recruited to Y449 on the IL-7Rα cytoplasmic tail and it activates Akt, resulting in increased cell survival[38].

The *IL7Rα* gene is located on chromosome 5p13.2 and is composed of eight exons that encode a 459 amino acid (a.a.) protein (Figure 6), with exons 1-5 encoding the extracellular domain, exon 6 encoding the transmembrane domain, and exons 7-8 encoding the cytoplasmic domain[38,39].

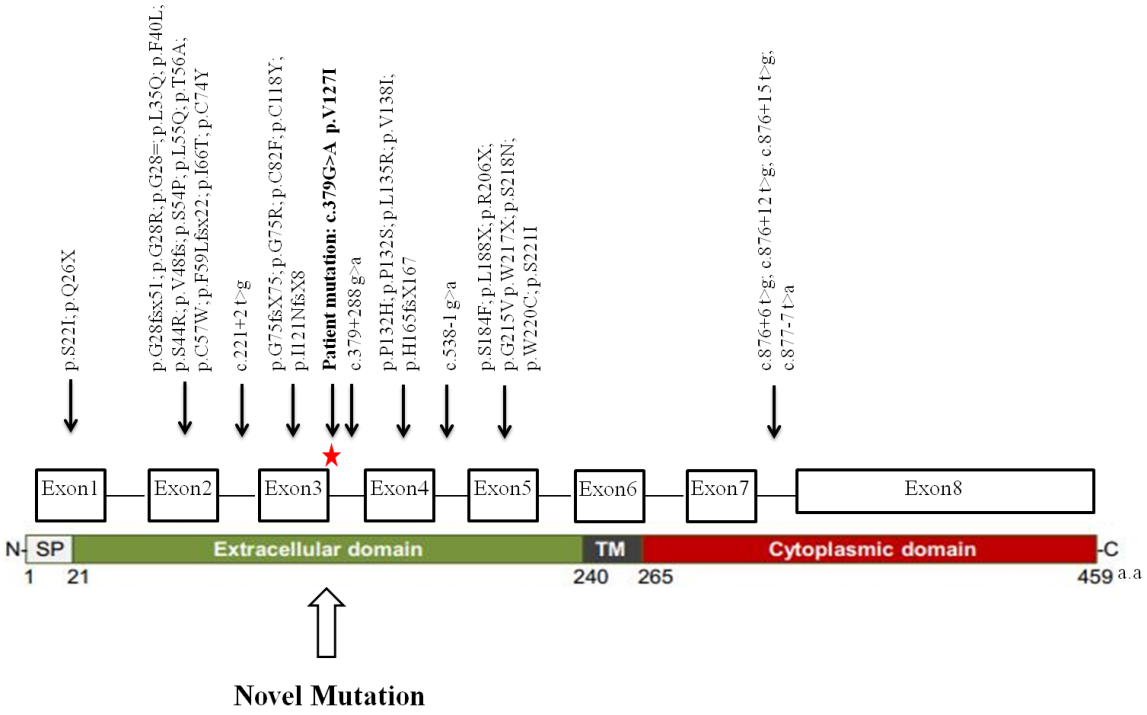

Figure 6. Schematic representation of the IL-7Rα RNA and the corresponding protein domains of the coding regions. Arrows indicate the location of all mutations in SCID patients described in the literature. The star indicates the location of the mutation identified in the patient under investigation in this study.

In addition to the plasma membrane-anchored IL-7Rα, a soluble cytoplasmic form of the receptor has been described that lacks the transmembrane domain because of alternative splicing out of exon 6[39,40]. The function of the soluble IL-7Rα is not clear but it is believed to enhance IL-7 signaling and increased levels of soluble IL-7Rα have been associated with autoimmune diseases such as multiple sclerosis[38,40].

Because of the important role IL-7Rα plays in the development and function of T cells, patients with deficient IL-7Rα expression or signaling have very few or completely absent T cells[27,41]. B cell counts are normal in the patients indicating that IL-7Rα facilitates, but is not crucial for B cell development, or that other compensatory mechanisms exist[37,42]. In addition, newborns, but not adults, can generate B cell progenitors from HSCs in cord blood (CB) independent of stimulatory signals from IL-7R[43,44]. Nevertheless, CB HSCs rely on Flt-3 ligand instead of IL-7 stimulatory signal, which might explain the presence of B cells in young patients with defective IL-7Rα[44]. These B cells will neither be fully developed nor functional because IL-7Rα is important for the proper development of B cells[45]. NK cell development and function is normal in the patients[46]. The patients are therefore classified as suffering from a $T^-B^+NK^+$ SCID phenotype[41,47] .

SCID patients with IL-7Rα deficiency exhibit severe clinical symptoms characterized by recurrent infections and autoimmune manifestations at an early age, and their disease course deteriorates to often become lethal without HSCT[34,48]. There are 53 SCID patients due to IL-7Rα deficiency described in the literature[38,42,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61]. Most of the mutations are located in the extracellular domain of IL-7Rα (Figure 6).

Table 1. SCID patients recorded in the literature review to have mutations in IL-7Rα

Patient	Clinical Manifestation	T Cell	T Cell Proliferation	Type of mutation	DNA mutation	Protein Mutation	Reference
1	N/A	Low	Defective	Homozygous	c.197 T>C c.412 G>A	p.I66T p.V138I	[49]
2	N/A	Low	Defective	Homozygous	c.651 G>A c.538-1 g>a (splice acceptor mutation)	p.W217X N/A	
3	N/A	N/A	N/A	N/A	c.197 T>C c.412 G>A	p.I66T p.V138I	[54]
4	N/A	N/A	N/A	Homozygous	c.166 A>G c.171 T>G	p.T56A p.C57W	
5	Rotavirus GE, disseminated BCG, PCP, rhinovirus infection, bronchiolitis obliterans with organizing pneumonia	Low	N/A	Heterozygous	c.65 G>T	p.S22I	[53]
6	RTI, recurrent oral candidiasis, local BCG disease	Low	N/A	Homozygous	c.562delC	p.L188X	
7	Intrauterine Omenn Syndrome, generalized exfoliative erythrodermia, edematous skin	Low	N/A	Homozygous	c.353 G>A	p.C118Y	[46]
8	Severe thrombocytopenic purpura	Low	N/A	Heterozygous	c.353 G>A c.361dupA	p.C118Y p.I121Nfsx8	
9	Erythroderma, hepatosplenomegaly, acute spongiform dermatitis with lymphohistiocytic infiltration of the dermis, Netherton's syndrome, persistent cytomegalovirus infection and autoimmunity (idiopathic thrombocytopenic purpura)	Low	N/A	Homozygous	N/A	p.C118Y	[51]
10	N/A	N/A	N/A	Heterozygous	c.361dupA c.616 C>T	p.I121NfsX8 p.R206X	[50]
11	Disseminated vaccine-acquired varicella (VZV) and vaccine-acquired rubella infections, respiratory syncytial virus (RSV) bronchiolitis, chronic cough, chronic oral mucositis and recurrent episodes of acute otitis media	Low	Defective	Hemizygous Heterozygous	c.361dupA Exon 3 deletion	p.I121NfsX8 p.G75fsx75	[61]
12	N/A	Low	Defective	Heterozygous Heterozygous	Exon 3 deletion c.221+2 t>g	p.G75fsx75 p.G28fsx35	[52]
13	Recurrent infections,RSV bronchio-litis, persistent rotavirus, candida, PJP, failure to thrive, Encephalopathy	Absent	Defective	Heterozygous Heterozygous	Exon 2-4 deletion c.76 C>T	p.G28fsx51 p.Q26X	
14	Failure to thrive, respiratory infection, Lymphopenia, RSV, PJP	Absent	N/A	Heterozygous Heterozygous	Exon 3 deletion c.221+2 t>g	p.G75fsx75 p.G28fsx35	
15	N/A	Absent	Defective	Heterozygous Heterozygous	Exon 3 deletion c.876+6 t>g, c.876+12 t>g, c.876+15 t>g (splice donor region/Exon 7 loss)	p.G75fsx75 p.K269fsx269	
16	N/A	None	Defective	Heterozygous Heterozygous	c.221+2 t>g c.76 C>T	p.G28fsx35 p.Q26X	
17	N/A	Absent	N/A	Heterozygous	c.76 C>T	p.Q26X	
18	N/A	N/A	N/A	Homozygous	c.493delC	p.H165fsx167	
19	N/A	N/A	N/A	Homozygous	c.493delC	p.H165fsx167	
20	Salmonella and Pneumocystis jiroveci infection, failure to thrive and pneumonia	Low	Normal	Heterozygous Heterozygous	c.353 G>A c.379+288 g>a (premature termination codon insertion)	p.C118Y N/A	[55]
21	Pneumocystis jiroveci pneumonia, FTT, oral candidiasis	Low	None	N/A	c.551 C>T	p.S184F	[56]
22	Family history of sister with SCID, no thymic shadow on chest X-ray, lymphopenia	Low	N/A	N/A	c.551 C>T	p.S184F	
23	Diarrhea, FTT, AIHA, pneumococcal sepsis, meningococcal meningitis, parainfluenza upper respiratory tract infection	Low	None	N/A	c.551 C>T	p.S184F	
24	Diarrhea, AIHA, ITP	Low	Defective	N/A	c.551 C>T	p.S184F	
25	Atopic eczema, *Staphylococcus aureus* Superinfection, remittent dermatitis, failure to thrive, recurrent pulmonary infections	Low	None	Heterozygous Heterozygous	c.177delT c.877-7 t>a (new acceptor splicing site)	p.F59Lfsx22 N/A	[57]

Table 1. SCID patients recorded in the literature review to have mutations in IL-7Rα

Patient	Clinical Manifestation	T Cell	T Cell Proliferation	Type of mutation	DNA mutation	Protein Mutation	Reference
26		N/A	None	Homozygous	c.223 G>A (splicing defect)	p.G75R	[42]
27		Low	None	Homozygous	c.223 G>A (splicing defect)	p.G75R	
28		Low	None	Homozygous	c.223 G>A (splicing defect)	p.G75R	
29		Low	None	Homozygous	c.353 G>A	p.C118Y	
30		Low	None	Homozygous	c.353 G>A	p.C118Y	
31		Low	None	Homozygous	c.638 C>T	p.R206X	
32		Absent	N/A	Homozygous	c.638 C>T	p.R206X	
33	Four patients (of the 16) had positive family history of SCID. Nine patients showed clinical symptoms of SCID, including fever, recurrent infections, *Pneumocystis jerovicii* pneumonia, prolonged diarrhea, and failure to thrive	Low	None	Homozygous	c.84 A>T (splicing defect)	p.G28=	
34		Low	None	Homozygous	c.186 T>A	p.L55Q	
35		Low	None	Heterozygous	c.154 C>A c.243 G>A	p.S44R p.C74Y	
36		Only maternal T cells	None	Heterozygous	c.154 C>A c.243 G>A	p.S44R p.C74Y	
37		Low	None	Homozygous	c.417 C>A	p. P132H	
38		Low	None	Heterozygous	c.165delTG	p. V48fs	
39		Low	N/A	Homozygous	c.104 G>A	p. G28R	
40		Low	None	Heterozygous	c.653 G>A c.682 G>C	p.S218N p.W220C	
41		Absent	None	Homozygous	c.462 T>G	p.L135R	
42	Persistent oral thrush, oral ulcers, and failure to thrive	Low	Defective	Homozygous	c.394 C>T	p.P132S	[47]
43	Oral candidiasis, failure to thrive, no lymph nodes	Low	Defective	Homozygous	c.394 C>T	p.P132S	
44	N/A	N/A	N/A	Homozygous	c.394 C>T	p.P132S	
45	persistent fever and cough, recurrent persistent infections, acute bronchiolitis, pneumonia, and oral thrush.	Low	N/A	Homozygous	c.616 C>T	p.R206X	[58]
46	Chronic diarrhea, FTT, adenovirus, CMV viremia chest infection bacterial tracheitis	Low		Homozygous	c.120 C>G	p.F40L	[48]
47	Neonatal fever, negative work-up, psoriatic-like rash	Low		Homozygous	c.120 C>G	p.F40L	
48	Normal	Low	N/A	Homozygous	c.120 C>G	p.F40L	
49	1 viral infection	Low	N/A	Homozygous	c.120 C>G	p.F40L	
50	Hepato-splenomegaly, diffuse erythroderma (Omenn) sepsis NEC totalis Laparotomy, short bowel resection, E. coli sepsis, candida infection	Low		Homozygous	c.120 C>G	p.F40L	
51	N/A	Low	N/A	Homozygous	c.644 G>T c.662 G>T	p.G215V p.S221I	[59]
52	N/A	Low	N/A	Heterozygous	c.104 T>A	p.L35Q	
53	Thrombocytopenia	Normal	Defective	Heterozygous Heterozygous	c.160T>C c.245G>T	p.S54P p.C82F	[60]

In my thesis research work, we studied a newborn female patient with auto-immune hemolytic anemia and recurrent infections since birth. We identified a mutation in the IL-7Rα and determined the effect of the mutation on gene function, RNA and protein expression, and T cell development and function. This work expanded the spectrum of SCID due to IL-7Rα deficiency and described a novel splicing defect that abolishes IL-7Rα surface expression. The patient underwent HSCT, therefore, we also studied the success of donor cell engraftment and function with time.

CHAPTER III

MATERIALS AND METHODS

A. Samples

Blood samples were collected in sodium-heparin tubes from adult healthy volunteers, the patient, and the parents following informed consents approved by the Institutional Review Board of the American University of Beirut Medical Center. The tests conducted took place at Immunology Research Laboratory of the Faculty of Medicine, American University of Beirut, Beirut, Lebanon.

B. Ficoll

Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque solution. Blood was layered onto 3ml of Ficoll solution in a 15ml sterile tube. Tubes were centrifuged at 2000 rpm (rounds per minute) for 30 minutes. Four separate layers are obtained in each tube; the serum, the buffy coat that consists of the PBMCs, the Ficoll-Paque media, and the erythrocytes along with granulocytes (Sigma-Aldrich Protocol). PBMCs were collected by using a sterile graduated pipette to remove the white buffy coat and placed in a sterile tube. The PBMCs are then washed 3 times using RPMI (10% FCS, 1% Peni/Strep, 1% L-glutamine, 1% Hepes).

Cells are counted using a hemocytometer and visualized under the microscope. The total number of cells is calculated using the following formula:
Total number of cells/ml = (number of cells/number of squares counted) * dilution factor * 10000.

C. Detection of surface markers by Flow Cytometry

200,000 PBMCs were used for extracellular markers staining. Antibodies used in this procedure were purchased from Biolegend. Four mixes of monoclonal antibodies were prepared, each mix being directed for a specified group of cells. Mix 1 is composed of a collection of antibodies specific for T cell detection that include anti-CD3-Percp (Cat# 300428), anti-CD4-PE (Cat# 317410), anti-CD8-AF700 (Cat# 344724), anti-CD45RA-PB (Cat# 304123), anti-CD45RO-APC (Cat# 304210), anti-CD31-PE/Dazzle red (Cat# 303130), and anti-CCR7-FITC (Cat# 353216) from Biolegend. Mix 2 is composed of antibodies directed against NK cells and includes anti-CD3-FITC (Cat# 300406), anti-CD16-PB (Cat# 302032), and anti-CD56-AF700 (Cat# 362522). Mix 3 consists of anti-CD19-Percp cy5.5 (Cat# 302230), anti-CD27-PE/Dazzle red (Cat# 356422), and anti-IgD-FITC (Cat# 348206) for B cell identification. Fourth mix is prepared to look for the expression of IL-7Rα and includes anti-CD3-FITC (Cat# 300406), anti-CD4-PE (Cat# 317410), anti-CD8-AF700 (Cat# 344724), and anti-CD127-PE/Dazzle red (Cat# 351336). The antibody mixes were then added to the cells and incubated for 30 minutes on ice (4 degrees Celsius). The cells were washed 1 time with FACS buffer and resuspended in 200µl FACS buffer to be read on FACS Aria flow cytometry/cell sorter, and analyzed using FlowJo.

D. T cell Proliferation assay

3 million cells are used for the proliferation assay. PBMCs were washed once with uncomplemented RPMI media and resuspended in 300µl of 1 µM carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich) for 6 minutes at 37°C. The staining reaction was halted by adding complemented RPMI media to the cells and

incubated for 10 more minutes at 37°C. The cells were washed once and then resuspended in complemented RPMI media to be distributed into the designed wells in the 96-well plate. Phytohaemmaglutinin (PHA; 2 µg/ml) and anti-CD3 (100ng/ml) were added to the cells as stimulants and cells were incubated for 5 days at 37°C. CD4 and CD8 cells were then stained with anti-CD4-PB (Cat# 317429) and anti- CD8-AF700 (Cat# 344724) for 30 minutes on ice, washed, and resuspended with FACS buffer and read on the FACS machine.

E. Regulatory T cell Staining

PBMCs were stained with surface markers anti-CD4-FITC (Cat#11004941, ebioscience) and anti-CD25-PE (Cat#302606, Biolegend) in 50µl FACS buffer. The mix was incubated for 30 minutes on ice then washed once with FACS buffer. Cells were then resuspended in Fix perm buffer, using eBioscience™ Fixation/Perm Diluent (lot# 2106782) and concentrate (lot# 2159394) from Thermo Fisher Scientific (1 volume concentrate with 3 volume of diluent) and incubated for 30 minutes at room temperature in the dark. Cells were then washed twice with 1x Permeabilization Buffer (lot#2140006, eBioscience) and resuspended in 100µl of 1x Permeabilization Buffer. 2µl of rat serum are added and incubated for 15 minutes at room temperature. Intracellular and intranuclear antibodies were added Foxp3-AF700 (lot# E103521632, ebioscience), CTLA-4-APC (Cat#349907, Biolegend), and Helios-PB (Cat#137220, Biolegend) and incubated with the cells for 45 minutes at room temperature. The cells were then washed with FACS buffer and resuspended in FACS buffer to be read on the FACS machine.

F. DNA extraction/ RNA extraction

Genomic DNA was purified using Puregene^{R} Blood Core Kit B (Lot No. 8850000033) from Qiagen and RNA extraction was done using TRIzol reagent (Sigma-Aldrich, MO, USA), according to the manufacturer's instructions.

G. Next Generation Sequencing (NGS)

A Targeted Gene Panel (TGP) of 300 genes involved in PID disorders were sequenced using Ion GeneStudio S5 System (ThermoFisher Scientific) based on the manufacturer's instructions[62] . In short, gDNA was purified from blood using Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). Custom primers were ordered through Ion AmpliSeq Designer (ThermoFisher Scientific, MA, USA) for the purpose of amplifying 6203 copies of intronic and exonic sequences. Genes of interest were amplified by the primers creating libraries that were amplified on ion spheres and loaded on 530 chips by the Ion AmpliSeq ™ Library Preparation Ion Chef ™ System (ThermoFisher Scientific). Homozygous and heterozygous Exonic and Splice site mutations were extracted from the sequencing machine in the form of excel files. Data analysis was conducted to study each mutation detected by the Ion Torrent sequencer and omit clinically unrelated mutations. Clinically relevant gene mutations were closely studied, and their pathogenicity was looked upon using several softwares designed to scale the severity of the mutation on a scale ranging from benign to severely deleterious. These softwares include SIFT, Polyphen, GnomAD, ClinVar, and CADD. SnapGene viewer software (GSL Biotech LLC, CA, USA) was used to visualize *IL7Rα* reference sequence obtained from the University of California Santa Cruz Genome Browser (UCSC).

H. Polymerase Chain Reaction (PCR)/ Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The extracted RNA was reverse-transcribed to cDNA using Reverse Transcription Applied Biosystems High Capacity cDNA Reverse Transcription Kit from Thermo Fisher Scientific (REF: 4368814) to obtain single stranded cDNA amplicons. Primers designed in our lab (Table 2) were used to obtain amplicons of cDNA and genomic DNA that was extracted beforehand.

Table 2. Name and location of the primers used, and amplicon size.

Gene	Forward Primer	Reverse Primer	Product size (bp)
IL-7Rα	FphIL7R(E3:10516) TGGGGCCCTCGTGGAGGTAAAG	RphIL7R(I3:10889) CACACCTGGGTTTGAAGATCC	374
IL-7Rα	FphIL7R(E2:4062) GAGACTTGGAAGATGCAGAACTG	RphIL7R(E4:14401) TCCTGGCGGTAAGCTACATCG	433

FphIL-7Rα and RphIL-7Rα primers were used to amplify segment exon 3 till intron 3 of *IL7Rα* gene. FphIL-7Rα (E2:4062) and RphIL-7Rα (E4:14401) were used to amplify the exon 2 to exon 4 region on the cDNA of *IL7Rα* gene, in a volume of 20 µl, 25 ng of gDNA or cDNA, 10 µM of each primer, 10x Taq buffer+ KCl (lot# 00099917) from Thermo Fisher Scientific, 25 mM $MgCl_2$ (lot# 00097122) Thermo Fisher Scientific, 25 mM of each dNTP, 0.1 µl of Taq DNA Polymerase (lot# 00059288) Thermo Fisher Scientific. The tubes were placed in the Thermal Cycler (Thermo Electron Corporation) using the following program: one cycle denaturation at 94°C for 3 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C degrees Celsius for 30 seconds, and elongation at 72°C for 30 seconds, and one cycle elongation at 72°C for 10 minutes. The PCR products obtained from IL-7Rα cDNA are run on

0.7% agarose gel and visualized by the use of ethidium bromide under ultraviolet light[54].

I. PCR product purification/ Sanger Sequencing

Genomic DNA and cDNA products were column purified using QIAquick^{R} PCR Purification Kit (Cat# 28104) from Qiagen. The purified gDNA products (using FphIL-7Rα (E3:10516) & RphIL-7Rα (I3:10889)) and cDNA products (using FphIL-7Rα (E2:4062) & RphIL-7Rα(E4:14401)) were then sent for Sanger Sequencing commercially.

CHAPTER IV

RESULTS

A. Patient history

The patient is a 1-year old female born to first degree consanguineous parents by Neonatal Vaginal Delivery (NVD) without perinatal complications. At the age of 6 months, she suffered from bronchiolitis. One month later she had a rotavirus infection followed by concomitant jaundice, nausea, and episodes of vomiting. The patient was reported to have a history of upper respiratory tract infections (URIs) and two episodes of oral thrush. In the first hospital admission, she was diagnosed with anemia with low hemoglobin (5.5 g/dl, normal range 10.8-12.6 g/dl)[63]. The patient was transfused with three units of blood for her anemia, two of which were given when admitted to the hospital and another one 2 months later. In a second hospital admission, her persistent anemia was investigated and she was suspected to suffer from SCID. She was treated with Bactrim to fight the recurrent infections, Rituximab to destroy the B cells that were suspected to produce autoantibodies against her RBCs resulting in AIHA, and Intravenous Immunoglobulin (IVIG) to replace her Abs and dilute any autoantibodies in her serum. Her history of immunization was up to date without any reported side effects.

The patient's family history is notable for four first degree cousins who died in infancy due to a suspected but undiagnosed immunodeficiency disorder. Therefore, the patient's clinical course and family history were highly suggestive of a PID, hence, we enrolled her in our study to identify the genetic and functional basis of her disease.

B. Clinical immunophenotyping

Before being referred to our laboratory, the patient had clinical immunophenotyping studies done on her blood during her hospitalizations (Table 3).

Table 3. Immunophenotype of the patient along with normal range for age[64,65,66].

Cell Surface Markers	Feb 14, 2020	March 4, 2020	Normal range
$CD45^+$ cells/µl	3,012 ↓	NA	3260-8840
$CD3^+$ cells/µl (%)	559 ↓ (18.57)	813 ↓ (36)	1850-5960 (52-77)
$CD3^+CD4^+$ cells/µl (%)	181 ↓ (6)	203 ↓ (9)	1140-3800 (31-56)
$CD4^+CD45RO^+$ %	NA	98.5 ↑	10.2-23.6
$CD4^+CD45RA^+$ %	NA	1.5 ↓	64-93
$CD4^+CD31^+CD45RA^+$ %	NA	9 ↓	47-79
$CD3^+CD8^+$ cells/µl (%)	375 ↓ (12.5)	587 (26)	540-1970 (12-27)
$CD8^+CCR7^+CD45RA^+$ %	NA	0.5 ↓	34-73
$CD8^+CCR7^+CD45RA^-$ %	NA	2.5 ↓	3-15
$CD8^+CCR7^-CD45RA^-$ %	NA	92 ↑	9-47
$CD8^+CCR7^-CD45RA^+$ %	NA	5↓	7-25
$CD4^+CD8^+$ cells/µl (%)	0.76 (0.03)	NA	
CD4/CD8 ratio	0.48	0.34	
$CD19^+$ cells/µl (%)	1206 (40.1)	294 ↓ (13)	549-1255 (15-28)
$CD16^+CD56^+$ cells/µl (%)	1219 ↑ (40.5)	1152 ↑ (51)	160-950 (3-15)

The patient's $CD45^+$ cell numbers were slightly below the normal range. Her $CD3^+$, $CD4^+$, and $CD8^+$ T cell numbers were considerably decreased for age, especially in the CD4 compartment. In addition, most of her T cells were of the memory phenotype ($CD45RO^+$) with a very low percentage of Recent Thymic Emigrant T cells (RTE; $CD4^+CD45RA^+CD31^+$). The patient had a very high percentage of effector memory $CD8^+$ T cells ($CD8^+CD45RA^-CCR7^-$) and decreased percentages of naïve ($CD8^+CD45RA^+CCR7^+$), central memory ($CD8^+CD45RA^-CCR7^+$), and exhausted effector memory ($CD8^+CD45RA^+CCR7^-$) CD8 T cells. The patient's B cell numbers were normal; however, she was treated with rituximab, an anti-CD20 monoclonal antibody, to deplete her B cells and treat her autoimmunity, resulting in a progressive

decrease to complete absence of her B cells. Her NK cell numbers were significantly elevated for age.

C. Immunophenotyping done in our laboratory

We were consulted on the patient to confirm her diagnosis and identify the genetic and functional basis of her suspected PID. First, we obtained a peripheral blood sample in tubes containing sodium heparin (Na-Hep) as anticoagulant, and ficolled the blood to isolate the peripheral blood mononuclear cells (PBMCs), which were used to identify the different T, B, and NK cell populations. Figure 7 describes the gating strategies that were used in the analysis. First, we gated on the lymphocytes followed by gating on the different markers for the various lymphocyte populations. In Figure 7A, we gated on the $CD3^+$ T cell population within the lymphocyte gate and identified the percentages of $CD4^+$ and $CD8^+$ T cells within the $CD3^+$ T cells. This showed that the patient (lower panels) had decreased percentage of lymphocytes (6% vs 44% in the healthy control, upper panels) with a decrease in the total percentages of $CD3^+$ and $CD4^+$ T cells and expansion of the $CD8^+$ population. We then determined the percentages of RTE T cells ($CD4^+CD45RA^+CD31^+$), a population of naïve cells that newly egressed out of the thymus, by gating on the $CD4^+$ T cells and checking the percentage of $CD45RA^+CD31^+$ (Figure 7B). This demonstrated that the RTEs were severely diminished in the patient as compared to the healthy control. Next, we gated on the $CD3^+$ lymphocytes to detect the percentages of naïve ($CD45RA^+$) and memory ($CD45RO^+$) T cells (Figure 7C) and found that the patient's T cells are mostly of the memory phenotype with absent naïve T cells. We then gated on the $CD8^+$ T cells and

checked for the percentage of naïve ($CD8^+CD45RA^+CCR7^+$), central memory ($CD8^+CD45RA^-CCR7^+$), effector memory ($CD8^+CD45RA^-CCR7^-$), and exhausted effector memory ($CD8^+CD45RA^+CCR7^-$) T cells (Figure 7D), and found that most of the peripheral $CD8^+$ T cells that the patient has are of the effector memory phenotype.

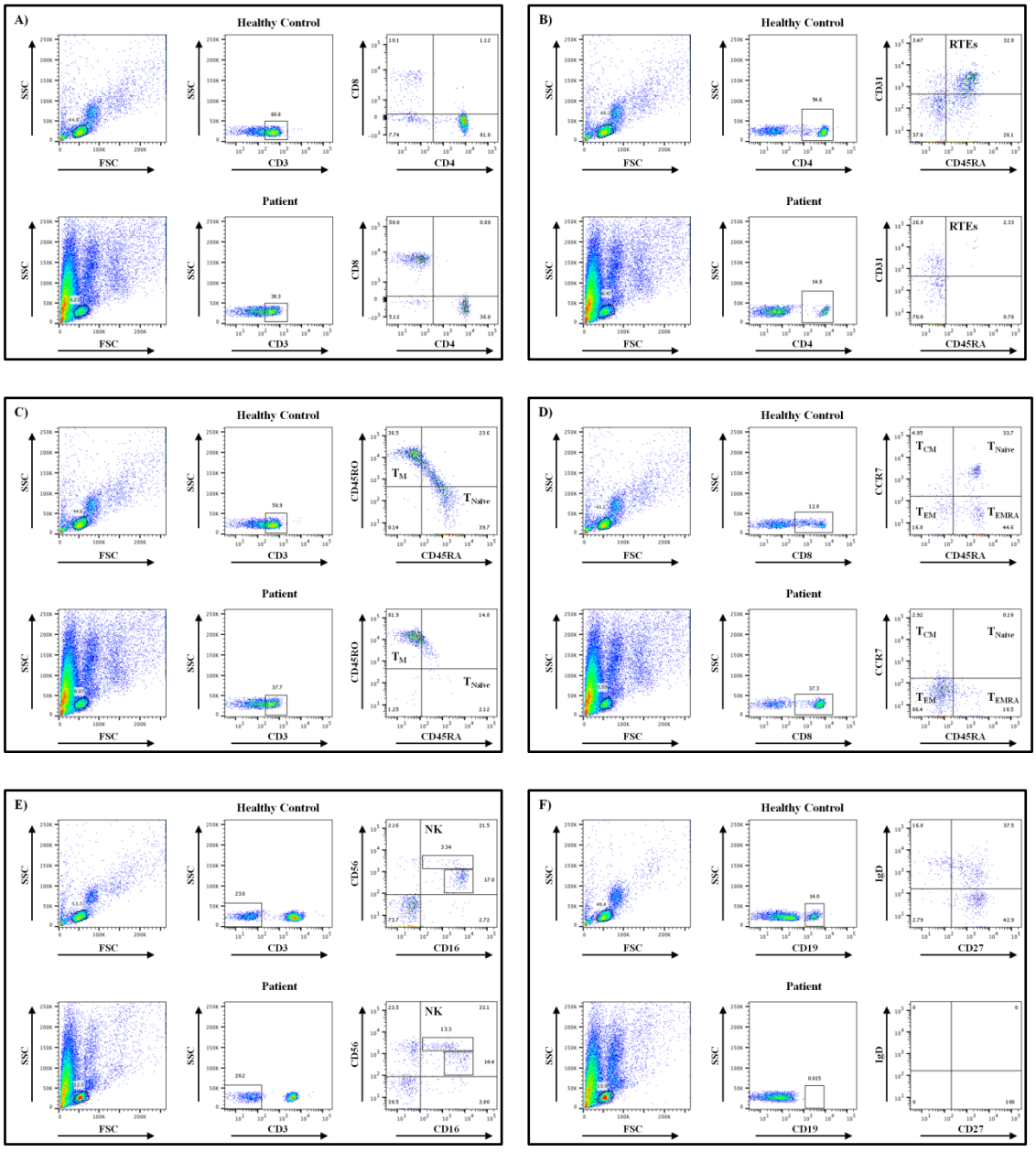

Figure 7. Flow cytometric representation of a selective PBMC population in the patient A) CD3-CD4-CD8 cells B) Recent Thymic Emigrants (RTEs) C) Memory and Naive T lymphocytes (T_M and $T_{Naïve}$) D) CD8 Effector Memory (T_{EM}), Central Memory (T_{CM}), Naïve ($T_{Naïve}$), and Terminally Differentiated Effector Memory cells Re-expressing CD45RA (T_{EMRA}) T cells E) Natural killer (NK) cells, and F) B cells.

To study the NK cell population, we gated on the $CD3^-$ cells and determined expression of CD56 and CD16, markers of NK cells (Figure 7E). We found that the patient had an increase in the percentage of NK cells, especially in the $CD56^{hi}$ naïve population that has a rather weak effector function[52]. We finally determined the percentages of B cells to identify the naïve ($CD19^+CD27^-IgD^+$), memory unswitched ($CD19^+CD27^+IgD^+$), and memory switched ($CD19^+CD27^+IgD^-$) populations, however, since the patient was on rituximab, we were not able to detect her B cells (Figure 7F).

This detailed immunophenotyping was repeated a month later, but the data is not shown here since it was identical to the above results.

D. T cell proliferation

The immunophenotyping results demonstrated that the patient has mainly a problem in the development of her T cells, however, it does not indicate if the few peripheral T cells she has are functional. Therefore, we tested the ability of patient's T cells to proliferate as an indicator of their function. PBMCs from the patient and a healthy control were stained with the cell proliferation dye carboxyfluorescein succinimidyl ester (CFSE) followed by stimulation with phytohemagglutinin (PHA) and anti-CD3 Abs. When T cells proliferate, CFSE will be distributed equally among the daughter cells resulting in a 50% decrease in the intensity of the dye with every round of cell division.

Five days after stimulation with PHA or anti-CD3, the cells were harvested and stained with anti-CD4 and anti-CD8 Abs and the proliferation of these T cells was measured. Figure 8 shows that both CD4 and CD8 cells isolated from the healthy control underwent several rounds of proliferation after stimulation with PHA and anti-

CD3, as determined by left shift in the peaks of the dye intensity on a histogram. However, the patient's T cells failed to proliferate under the same conditions (Figure 8).

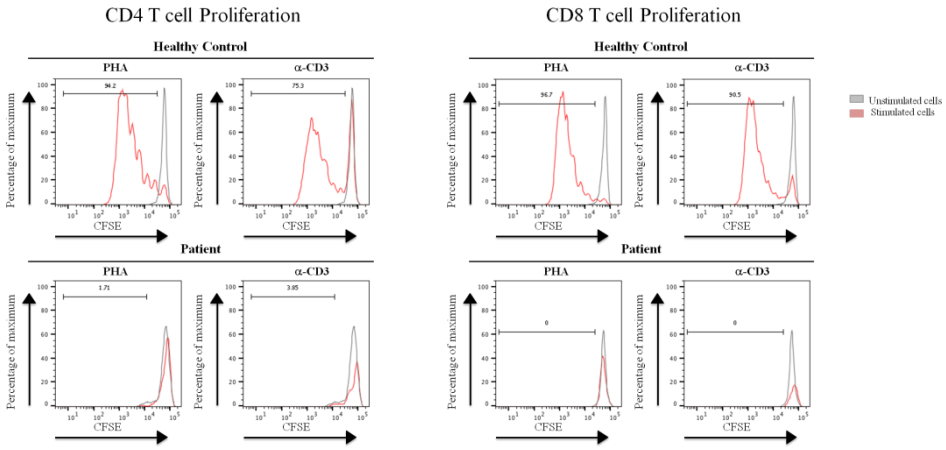

Figure 8. Histograms showing CD4 and CD8 T cell proliferation as measured by CFSE dilution.

These results demonstrated that the few peripheral T cells the patient has are not functional, confirming that she is a phenotypic and functional SCID patient.

E. Next Generation Sequencing Analysis

Having confirmed that the patient suffers from SCID, we went on to identify the genetic etiology of her disease using Next Generation Sequencing (NGS). Before being referred to our laboratory, genetic tests were conducted in France on DNA isolated from the patient, which reported that the patient does not harbor homozygous mutations in any of *RAG1*, *RAG2*, and *DCLRE1C* genes.

To further study her disease at the genetic level, we performed NGS to sequence a panel of 300 genes known to be involved in the development of PID using the ion torrent machine located at the Pillar Genomic Institute of the Faculty of Medicine at

AUB. This revealed a homozygous conversion of a guanine (G) to alanine (A) at position 379 (c.379G>A) in exon 3 of the coding sequence of the *IL7Rα* gene, with a high coverage of 465x. This missense mutation resulted in the conversion of a valine (V) to an isoleucine at position 127 (p.V127I) of the IL-7Rα protein (Figure 9A). This mutation is predicted to be "deleterious" by the softwares SIFT (score of 0.01) and CADD (score of 33), and "possibly damaging" by Polyphen (score of 0.86).

F. Sanger Sequencing of exon 3 from gDNA of the patient

To confirm the presence of the mutation detected by NGS, we used Sanger sequencing of gDNA isolated from the patient, her parents, and a healthy control. The region surrounding the mutation was amplified by Polymerase Chain Reaction (PCR) using a forward primer that binds on exon 3 and a reverse primer that binds on intron 3, then the amplified product (374 bps) was sent for Sanger sequencing commercially. The chromatograms shown in Figure 9B confirm the homozygous G>A mutation in the patient (red box). In addition, both parents are heterozygote for the same mutation, whereas the control has the reference G at this position. These data confirm the mutation detected by NGS and demonstrate that both parents are carriers for this mutation.

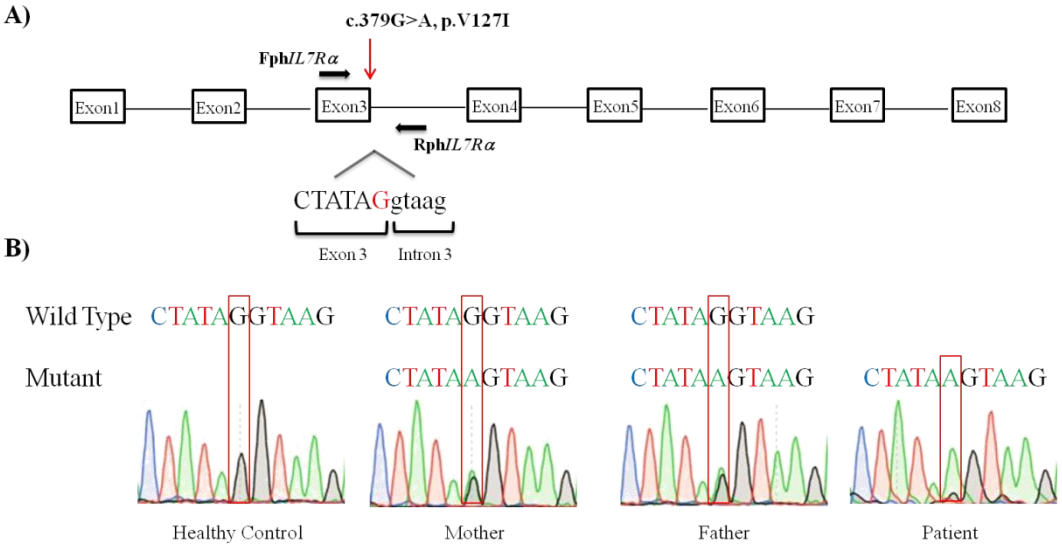

Figure 9. Chromatogram representation of Sanger sequencing on IL-7Rα gDNA. A) Genomic organization of the *IL7Rα* gene locus with the arrows indicating the location of the primers used in the PCR amplification strategy. B) Chromatograms showing the Sanger sequencing results of exon 3 amplified by PCR using gDNA isolated from a healthy control, mother, father, and the patient.

G. Expression of IL-7Rα on the surface of T cells from the patient

To determine the effect of the V127I mutation on the expression of IL-7Rα, we determined the levels of IL-7Rα on the surface of T cells using flow cytometry. Figure 10A shows normal expression of IL-7Rα on 80% of the healthy control $CD3^+$ T cells. Similarly, 72% and 79% of the mother's and father's $CD3^+$ T cells express the IL-7Rα, respectively, whereas only 6.5% of the patient's $CD3^+$ T cells express the IL-7Rα. Furthermore, the level of IL-7Rα expression on the parents' $CD3^+$ T cells was around 50% of that observed in several HCs (Figure 10B) indicating that the homozygous mutation is deleterious as it abolishes IL-7Rα protein expression in the patient, and reduces the expression of IL-7Rα by half in the parents since they harbor 1 wild type and 1 mutant allele of IL-7Rα.

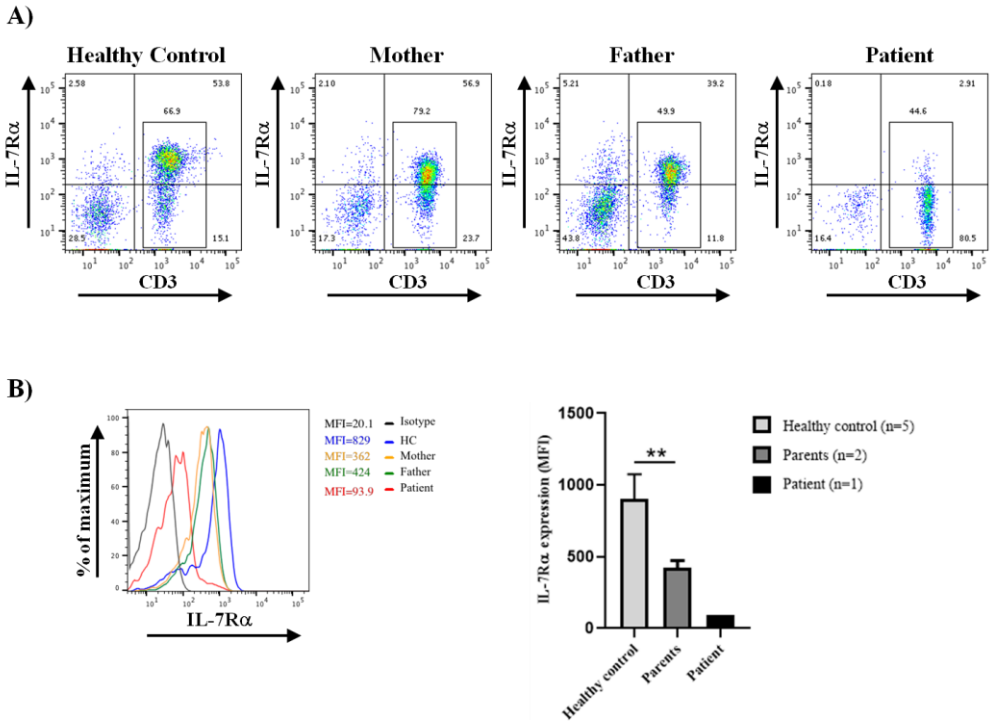

Figure 10. Effect of the V127I mutation on the expression of IL-7Rα A) Flow cytometry dot plot analysis of IL-7Rα cell surface staining on $CD3^+$ T cells from a healthy control, mother, father, and patient. B) Histogram and bar graph representation of IL-7Rα levels on $CD3^+$ T cells from a healthy control, mother, father, and the patient. The numbers on the right of the histogram represent the Mean Fluorescence Intensities (MFI) of IL-7Rα expression on the surface of T cells. A non-specific isotype Ab was used as a negative control. **, $p<0.001$. HC: Healthy control

H. Effect of the c.379G>A mutation on exon 3-exon 4 splicing

We wanted to determine how the c.379G>A missense mutation completely abolishes protein expression. Since the mutated residue is the last nucleotide at the 3'end of exon 3, we hypothesized that mutation of the G>A could affect the splice donor site of exon 3, resulting in aberrant splicing of exon 2 to exon 4 bypassing exon 3. To test this hypothesis, we designed a PCR strategy to amplify exons 2 to 4 using cDNA from the patient and determined the size of the amplified product as well as its sequence.

RNA was extracted from the PBMCs isolated from the patient, her parents, and a healthy control. RNA was reverse transcribed to cDNA, and the cDNA was used in a PCR strategy with primers that bind in exon 2 and 4, as shown in Figure 11A. Amplification of exons 2 to 4 are expected to give a 433 bp wild type band. If the mutation detected at the end of exon 3 affects splicing of exon 3 to 4, then the alternative splicing of exon 2 to 4 would result in a 275 bp mutant band that lacks exon 3.

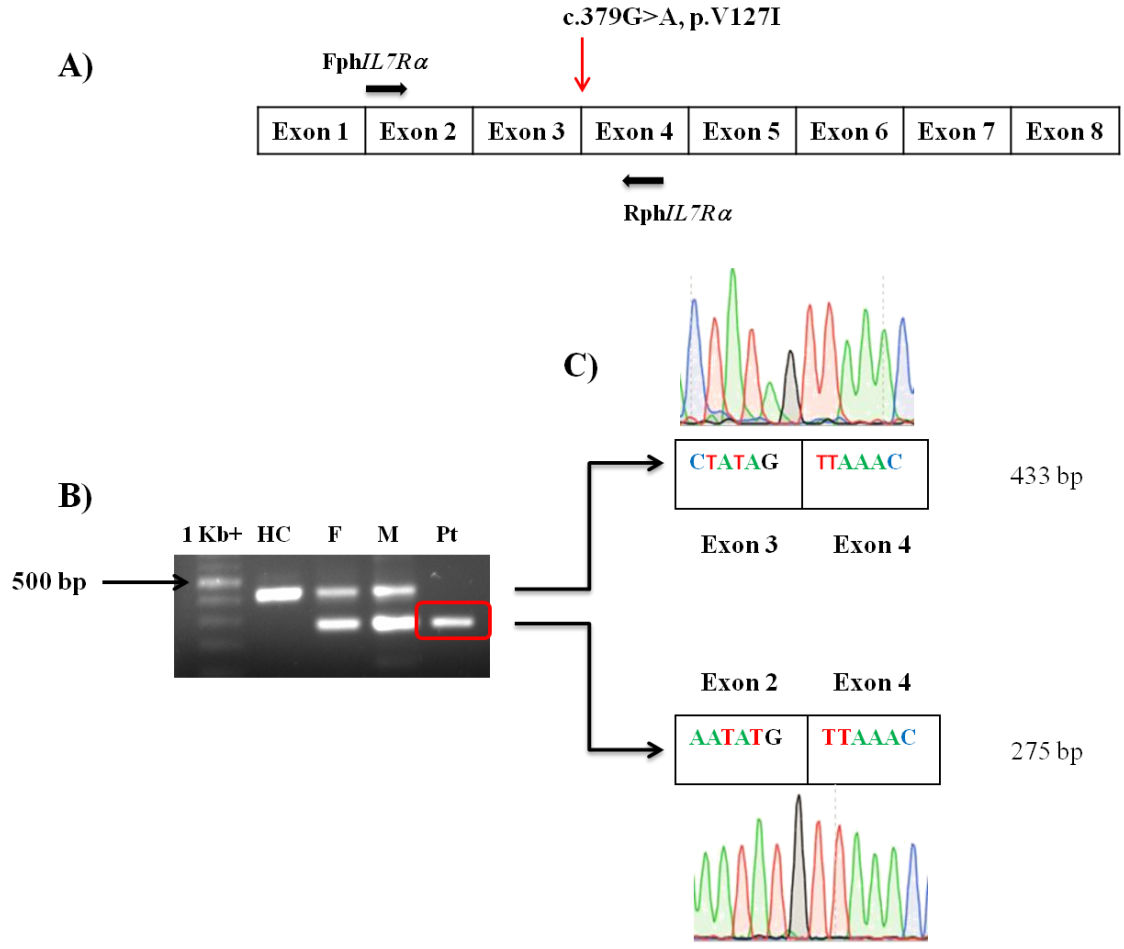

Figure 11. Aberrant splicing of exons 2 to 4 in the patient's cDNA. A) Schematic representation of the *IL7Rα* coding sequence with the arrows indicating the location of the primers used in the PCR amplification strategy. B) Agarose gel showing the nature

and size of PCR amplification products. C) Sanger sequencing results of the PCR products obtained, with boxes showing the sequences at the junction of exons 3 and 4, or exons 2 and 4. Healthy control, HC; Father, F; Mother, M; Patient, Pt.

Using this PCR strategy, we obtained the expected wild type 433 bp band using cDNA prepared from the healthy control (Figure 11B, HC). On the other hand, we obtained a smaller band (275 bp mutant band) using cDNA from the patient (Figure 11B, Pt), suggesting that the smaller band obtained lacks exon 3. Using cDNA prepared from both parents, the results of the gel showed a wild type and a smaller mutant band reflecting the heterozygous nature of the mutation in the parents that results in 2 RNA/cDNA products.

To prove that the smaller band detected in the patient results from alternative splicing of exons 2 to 4, the PCR products were purified by column purification and subjected to Sanger sequencing commercially. Sequencing of the PCR product purified from the healthy control cDNA showed normal splicing of exons 3 to 4, as shown in the chromatogram overlaying the illustration of the wild type band, which represents the last 6 nucleotides from exon 3 and the first 6 nucleotides of exon 4 (Figure 11C). However, sequencing of the of the PCR product purified from the patient cDNA demonstrated aberrant splicing of exons 2 to 4, as shown in the chromatogram underlaying the illustration of the mutant band, which represents the last 6 nucleotides from exon 2 and the first 6 nucleotides of exon 4.

Sanger sequencing of the mixed PCR products obtained using the parents' cDNA (Figure 11B) gave normal exon 2 sequence followed by 2 overlapping sequencing peaks due to the simultaneous sequencing of exon 3, encoded by the wild type cDNA, and exon 4, encoded by the mutant cDNA. Figure 12 illustrates the chromatograms obtained after sequencing the cDNA from the healthy control, parents,

and patient, which demonstrates that the healthy control has normal splicing of exon 3 to 4, and that the patient has aberrant splicing of exons 2 to 4. The chromatograms of both parents show a mix of wild type and mutant cDNA of IL-7Rα revealed by the normal sequence of exon 2 followed by the sequence of exon 3 and exon 4 reflecting their heterozygosity.

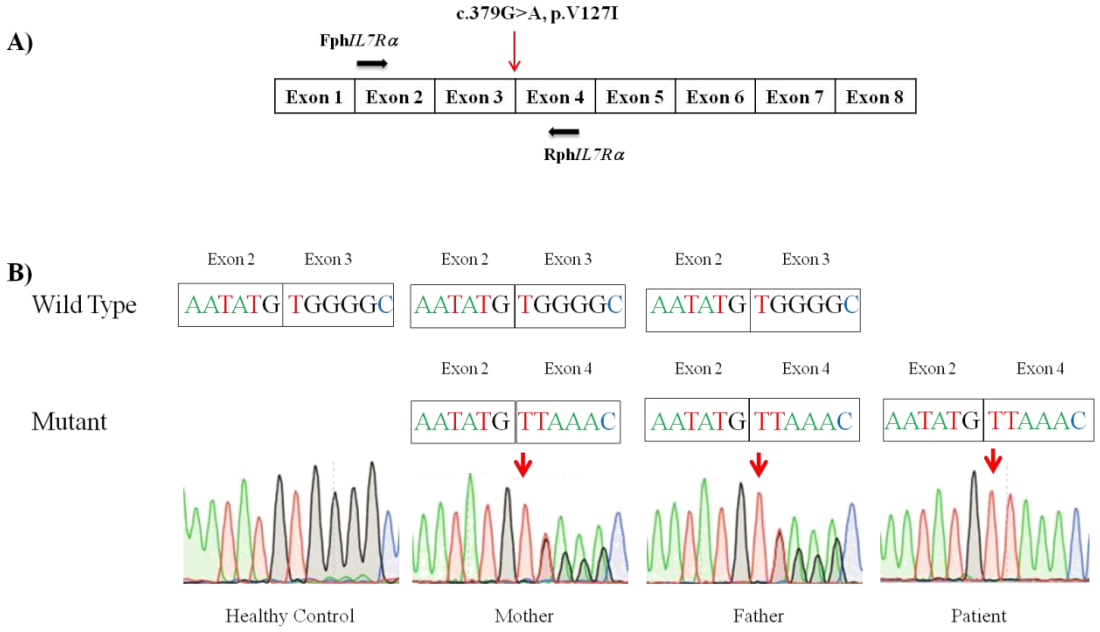

Figure 12. Effect of the c.379G>A mutation on the splicing of *IL7Rα* exon 3 to exon 4. A) Schematic representation of the *IL7Rα* coding sequence with the arrows indicating the location of the primers used in the PCR amplification strategy. B) Chromatograms showing the Sanger sequences of the exon boundaries.

I. Effect of the p.V127I mutation on the IL-7Rα protein sequence

The last two nucleotides in exon 2 (TG) form a codon with the first nucleotide of exon 3 (T) to encode the amino acid (a.a.) cysteine (TGT). In addition to that, the last nucleotide of exon 3 (G) forms a codon with the 2 nucleotides starting exon 4 (TT) to encode the a.a. valine (GTT). In the patient, loss of exon 3 results in the splicing of exon 2 to exon 4, as described earlier. When this takes place, the last two nucleotides in

exon 2 (TG) will now form a new codon with the first nucleotide in exon 4 (T) to also encode cysteine (TGT), however, the neighboring T will now combine with the AA to form a new stop codon (TAA) that will result in arrest of IL-7Rα protein translation on the ribosome. The whole sequence of nucleotides following this stop codon is shifted causing insertions of several stop codons throughout exons 4-8 of the IL-7Rα mRNA. The protein that might be expressed will only be encoded by exons 1 and 2, while the major part is lost. We believe that this truncated protein will most likely be degraded inside the cell.

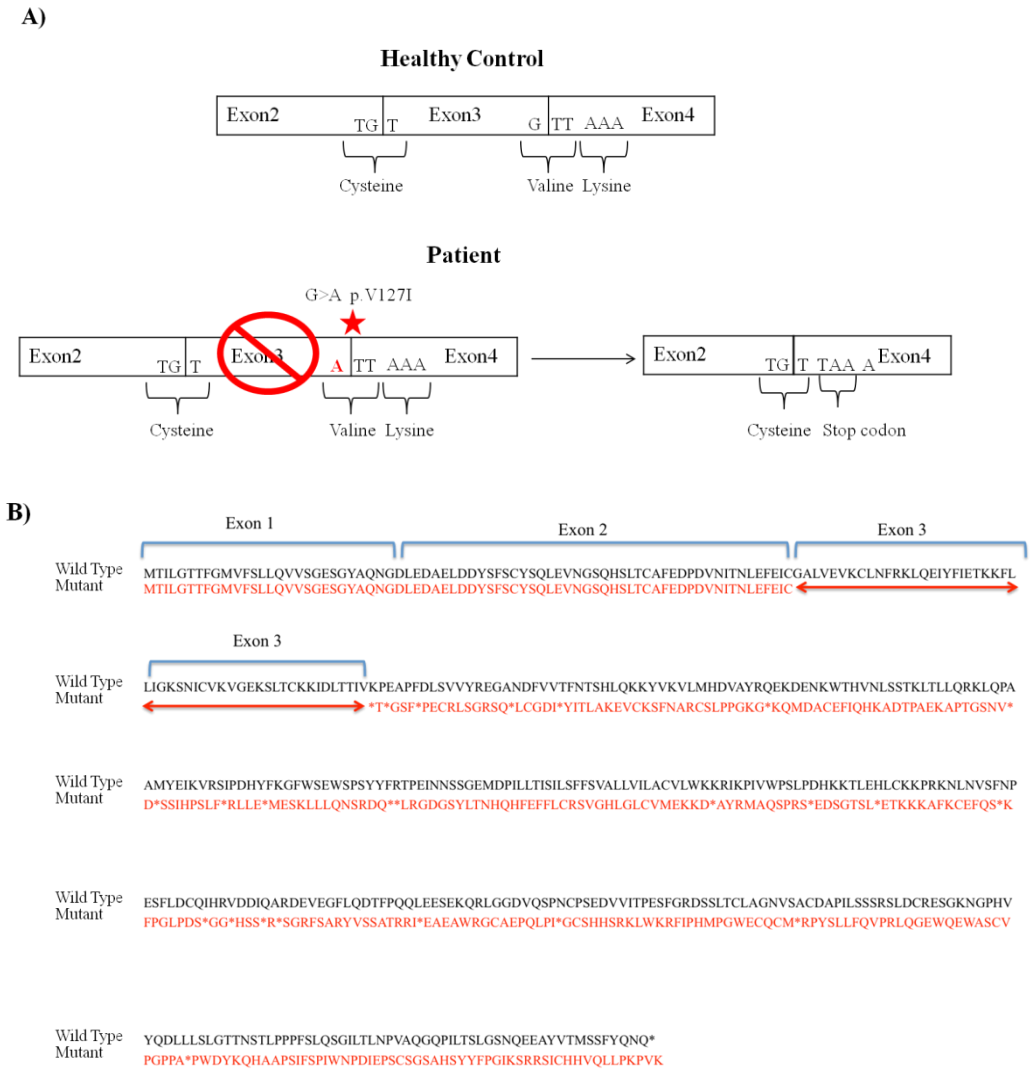

Figure 13. Effect of c.379G>A mutation on the IL-7Rα protein sequence. A) Schematic representation for the effect of aberrant exon 2 to 4 splicing on the coding frame of the

IL-7Rα protein. B) Sequence alignment of wild type IL-7Rα protein with the predicted out-of-frame mutant IL-7Rα protein sequence in the patient.

These results demonstrate that the c.379G>A mutation abolished normal splicing of the *IL7Rα* exon 3 to exon 4, resulting in an abnormal coding RNA and a frameshift that takes the IL-7Rα protein out of frame, causing loss of normal IL-7Rα expression on the surface of T cells.

J. Post-transplant studies

The patient underwent hematopoietic stem cell transplantation (HSCT) using her HLA-matched grandmother as donor. Ten months after transplant we received peripheral blood from her to determine the efficiency of donor cell reconstitution.

1. Immunophenotyping

After transplant, the percentages of lymphocytes, $CD3^+$ and $CD4^+$ T cells increased significantly in the patient (Figure 14A). In addition, the percentage of RTE increased from around 2% of CD4 cells before transplant, to 15% post-transplant (Figure 14B) and her T cells ranged from naïve to memory, as compared to only memory T cells before transplant (Figure 14C). On the other hand, her CD8 percentage were still elevated, and most of them were still effector memory cells with very few naïve cells (Figure 14D), similar to pre-transplant. Donor NK cell percentages were low because the HSC regimen might have ablated her own NK cells and the donor cells has not fully emerged yet (Figure 14E), while donor B cells were absent (Figure 14F) because the patient was still receiving rituximab as she was suffering from AIHA even after the transplant.

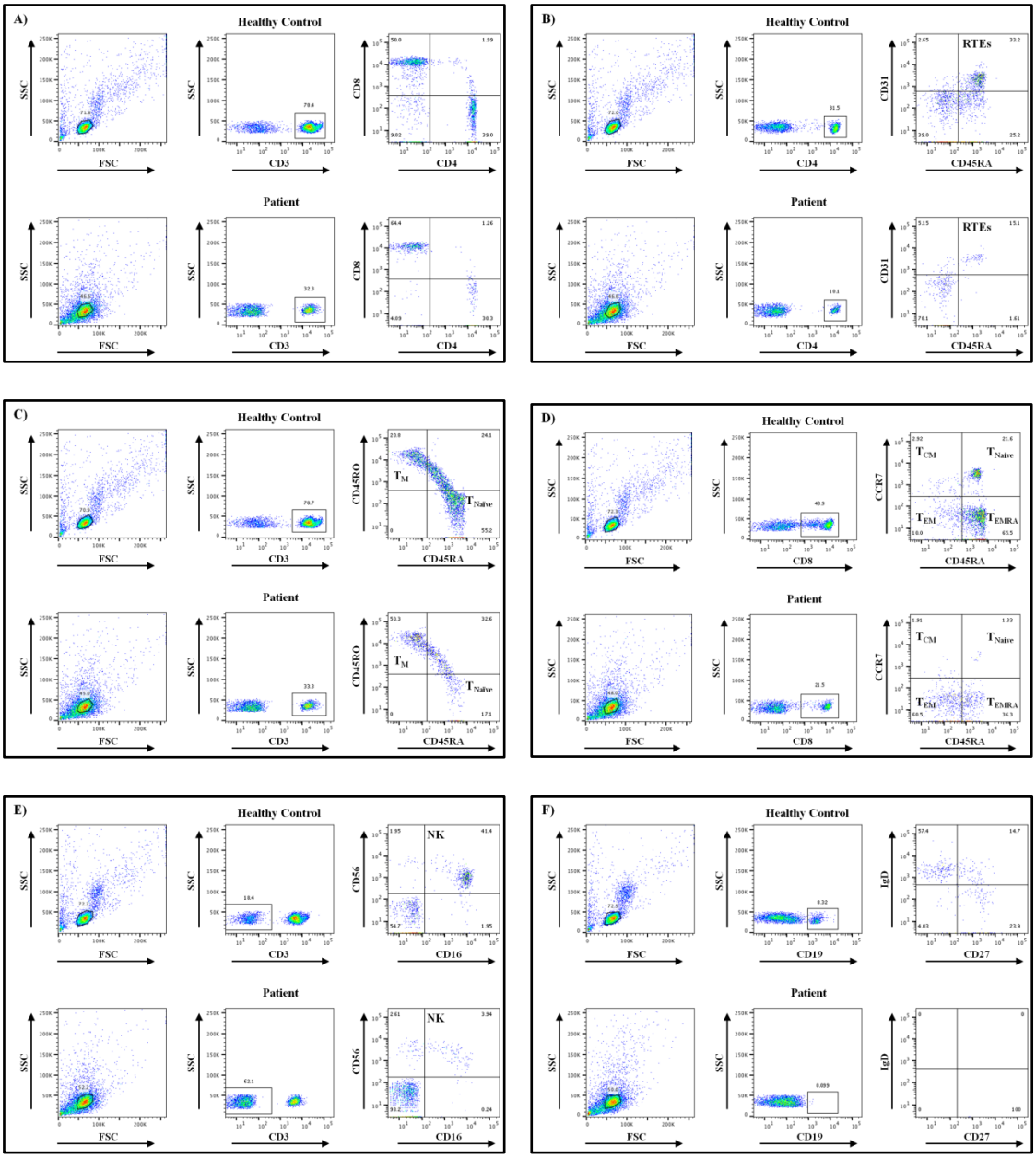

Figure 14. Flow cytometric representation of a selective PBMC population in the patient after HSCT. A) CD3-CD4-CD8 cells B) Recent Thymic Emigrants (RTEs) C) Memory and Naïve T lymphocytes (TM and TNaïve) D) CD8 Effector Memory (TEM), Central Memory (TCM), Naïve (TNaïve), and Terminally Differentiated Effector Memory cells Re-expressing CD45RA (TEMRA) T cells E) Natural killer (NK) cells, and F) B cells.

2. Regulatory T cell (Treg) Staining

Because of her AIHA, we determined the percentages of regulatory T cells (Tregs) that the patient has generated following transplant, by gating on $CD4^+$ T cells and determining the nuclear expression of the Treg marker Foxp3, a transcription factor important for the development and function of Tregs. Figure 15 shows normal production of Treg cells ($CD4^+Foxp3^+$) in the patient. In addition, the patient's Tregs are functional as they express high levels of the surface protein CD25, and the intracellular proteins CTLA-4 and Helios (Figure 15 lower panels) which are important to mediate the inhibitory function of Tregs.

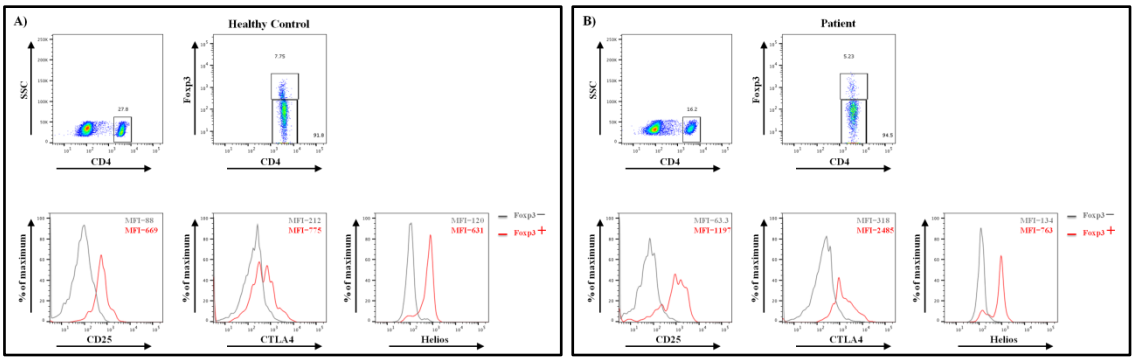

Figure 15. Treg staining by flow cytometry for A) Healthy control B) Patient.

3. IL-7Rα expression on T cells from the patient

To determine the efficiency of donor HSC reconstitution, we used flow cytometry to determine the percentage of $CD3^+$, $CD4^+$, and $CD8^+$ T cells that express the IL-7Rα, which are of donor origin. Figure 16A & B show that the IL-7Rα is expressed on 44% of the patients' $CD3^+$ T cells as compared to 79% in the healthy control, and that this expression is mainly on $CD4^+$ T cells (79% in the patient vs 97% in the healthy control), whereas only 15% of the patient's $CD8^+$ cells expressed the IL-

7Rα as compared to 59% in the healthy control. This data indicates that, at the time of this experiment, there is better development of $CD4^+$ than $CD8^+$ T cells. Furthermore, gating only on the IL-$7R\alpha^+$ cells from each population (CD3, CD4, and CD8), we found that the levels of IL-7Rα expression was around 50% in the patient as compared to the control (Figure 16C).

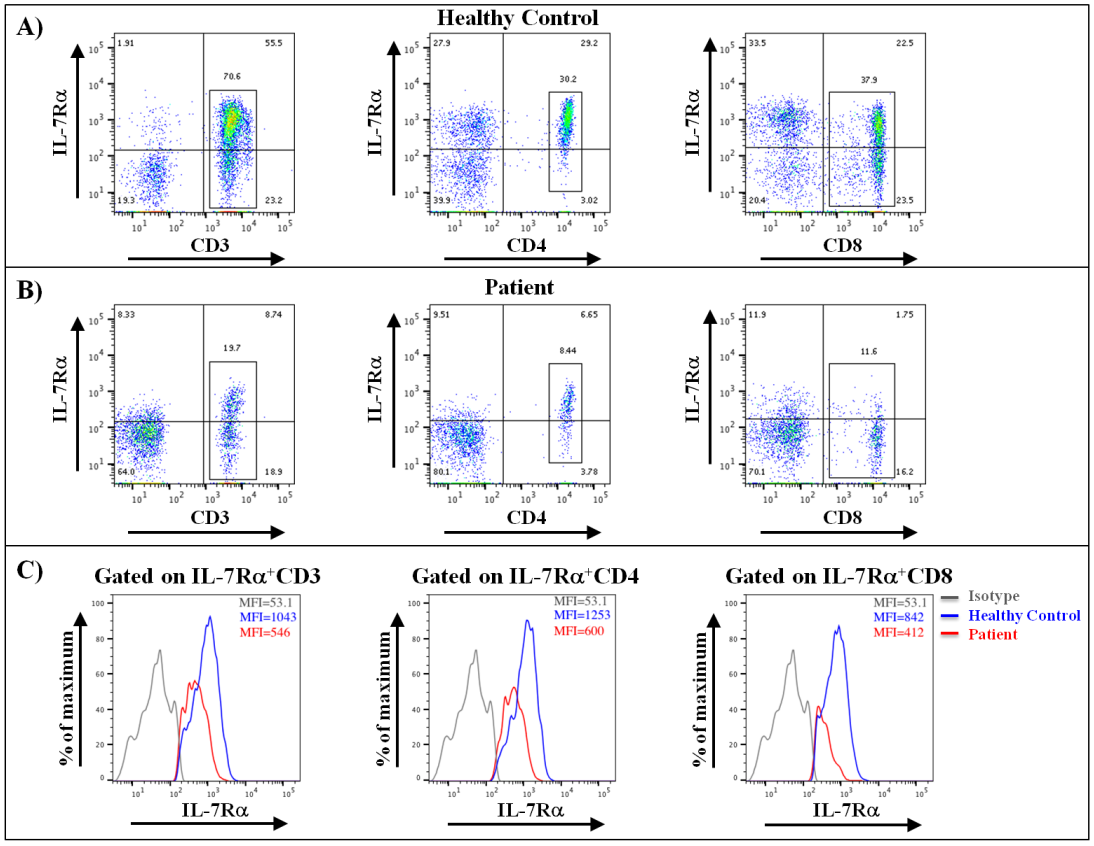

Figure 16. Flow cytometry analysis of IL-7Rα cell surface staining after HSCT on CD3+, CD4+ and CD8+ cells for a (A) healthy control and (B) the patient. C) Histogram representation showing IL-7Rα expression of healthy control and patient gating on $CD3^+$, $CD4^+$, and $CD8^+$ T cells.

4. Proliferation

To determine if the reconstituted T cells are functional, we checked their ability to proliferate following stimulation (Figure 17). Around 55% and 49% of the patient's CD4 T cells proliferated when stimulated with PHA and anti-CD3 mAbs, respectively,

as compared to 76% and 49% of the healthy control CD4 T cells (Figure 17A). On the other hand, the proliferation of CD8 T cells was decreased in the patient with only 34% and 24% when stimulated with PHA and anti-CD3 mAbs, respectively, as compared to 80% and 75% of the healthy control CD4 T cells (Figure 17B). This indicates that the engrafted CD4, and, to a much lesser extent CD8, T cells can proliferate in response to stimuli.

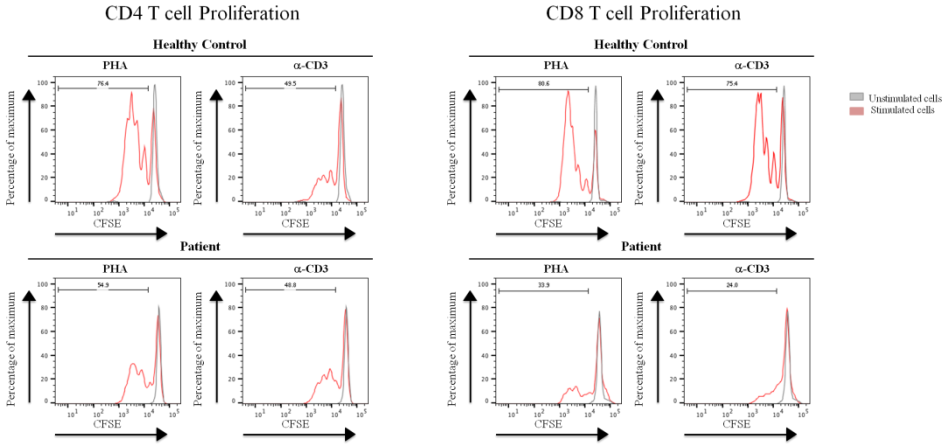

Figure 17. Histograms showing CD4 and CD8 T cell proliferation as measured by CFSE dilution after HSCT.

CHAPTER V

DISCUSSION

In this study, we describe a patient of consanguineous parents with $T^-B^+NK^+$ SCID due to a deleterious homozygous c.379G>A mutation in *IL7Rα* coding sequence that results in aberrant RNA splicing, loss of protein expression, and poor T cell production and function. The patient was successfully transplanted at AUB-MC with HLA-matched allogeneic HSCs using her paternal grandmother as donor, resulting in partial reconstitution of her T cell development and function.

The patient is the only child of first-degree consanguineous parents and has a family history of four first-degree cousins dying at early age due to a suspected but unconfirmed immunodeficiency. She developed recurrent upper respiratory tract infections, oral thrush, and severe AIHA since birth. Because her clinical and family histories were very suggestive of a PID, she was suspected and investigated early on by her treating physician, and enrolled in our PID study for diagnosis and HSC.

The patient was admitted to the hospital on several occasions where she was treated with bactrim for the recurrent infections, rituximab (anti-CD20 mAb) to destroy her B cells and eliminate autoantibodies against her RBCs that resulted in AIHA, and intravenous immunoglobulin (IVIG) to replace her Abs and dilute whatever autoantibodies she might have. However, her most critical clinical emergency was the AIHA as her hemoglobin levels were dangerously low. This early development of autoimmunity highlights the immune dysregulation observed in patients with SCID, which is often one of the earliest manifestations of their PID and could confuse the diagnosis[67].

During her early hospital admissions, clinical immunophenotyping showed decreased absolute numbers of peripheral T cells with normal to elevated numbers of B and NK cells. However, her B cell numbers steadily decreased and became undetectable because she was treated with rituximab.

A detailed immunophenotyping done in our laboratory confirmed the low percentage of lymphocytes, $CD3^+$, and $CD4^+$ T cells with expansion of her $CD8^+$ T cells. In addition, her RTEs were almost absent indicating defective thymic output, and suggesting that whatever T cells she has in the periphery likely results from the expansion of a few T cell clones that escaped selection in the thymus, or from maternal T cell engraftment. Moreover, her peripheral T cells were mostly memory exhausted cells that failed to proliferate *in vitro* when stimulated with PHA and anti-CD3 mAbs. The patient had a high percentage of effector memory CD8 T cells (T_{EM}) that are known to play an essential role in protective memory by going to the sites of inflammation in the periphery and actively fighting infections[68]. Her NK cell numbers were high, however, around 50% were $CD56^{hi}$ indicating that they are a naïve population that has a weak effector function, as compared to $CD56^{lo}$ NK cells that are more developed with higher cytolytic activity[69]. The healthy control's NK cell population was mostly composed of $CD56^{low}$ effector NK cells. Her B cells were undetectable because she was treated with rituximab that depletes B cells. When we initially received blood from the patient, we did not quantify her Tregs due to the low number of cells we obtained as the patient was lymphopenic. However, we believe that the early manifestation of her autoimmunity in the form of AIHA was likely associated with absent or defective Treg cells[70,71]. In conclusion, her clinical phenotype and laboratory data demonstrated that

she is a phenotypic and functional $T^-B^+NK^+$ SCID, therefore we pursued genetic testing to uncover the genetic etiology of her disease.

Next generation sequencing (NGS) analysis of the patient's genomic DNA using a targeted gene panel approach revealed a homozygous c.379G>A mutation in the *IL7R*α coding sequence. This mutation is consistent with the immunological phenotype of $T^-B^+NK^+$ SCID in the patients because *IL7R*α is essential for T cell development and function[31]. In addition, the c.379G>A mutation was confirmed in the patient using Sanger sequencing, and both parents were found to be carriers for the same mutation, and hence have a 25% chance of transferring this mutation in the homozygous form to their children. The genetic data was consistent with the clinical and immunological phenotype of the patient; therefore, we pursued cellular and molecular studies to elucidate the role of this mutation in disease development.

Flow cytometry was used to determine the effect of the mutation on the expression of IL-7Rα on the patient's T cells. This revealed that the vast majority (93.5%) of the patient's $CD3^+$ T cells did not express IL-7Rα and demonstrated that the mutation was deleterious. Only 6.5% of the patient's $CD3^+$ T cells expressed IL-7Rα indicating that they might be maternal cells transferred to the patient *in utero*. This suggestion is supported by a study done on 121 SCID patients where 40% were found to have transplacentally acquired maternal cells in the periphery[72]. In addition, neither of the patient's CD4 and CD8 T cells proliferated when stimulated with PHA and anti-CD3 mAbs, giving further evidence for the role of the mutation in abolishing T cell function. Therefore, the absence of IL-7Rα expression on T cells from the patient, coupled with defective T cell development and function provided definite evidence for the pathogenic nature of the mutation and its pathologic effect on the patient's health.

We were intrigued to understand how the c.379G>A missense mutation causes complete loss of protein expression. On a closer look at the location of the mutated nucleotide, we noticed that it was the last residue at the 3' end of exon 3 in the *IL7Rα* gene, so we suspected that it might be affecting the adjacent intronic splice donor site of exon 3. We envisaged that three scenarios could occur: 1) the mutation would not interfere with the splicing of the *IL7Rα* RNA, 2) the splicing machinery will fail to splice exon 3 into exon 4, thus a portion of intron 3 will be incorporated into the RNA, or 3) the splicing machinery will alternatively splice exon 2 to exon 4 resulting in an RNA that lacks exon 3. Using PCR from cDNA prepared from the patient, we demonstrated that the mutated residue totally abolished splicing of exon 3 to exon 4. Instead, we found a single RNA product generated by the complete splicing of exon 2 to exon 4, resulting in RNA that lacked exon 3, which, if translated, would encode an out-of-frame protein with premature termination of translation. This suggests that the splicing machinery is capable of splicing across exons; however, it is tightly regulated to maintain proper splicing and generate a productive RNA that can be translated into the functional protein. The parents of the patient are heterozygous for the same mutation, and they expressed a wild type and a mutant RNA species, thus they would express half the number of IL-7Rα proteins on the surface of their T cells, which correlates with the 50% decrease in MFI of IL-7Rα on their T cells detected by flow cytometry. These results identify the molecular effect of the mutation on the *IL7Rα* RNA integrity, and the mechanism of loss in IL-7Rα protein expression on the patient's cells.

The patient underwent allogeneic HSCT from her HLA-matched paternal grandmother with a mild conditioning regimen resulting in around 70% chimerism.

Blood samples were collected from the patient ten months after transplant to assess the engraftment and function of donor cells. Immunophenotyping analysis demonstrated an increase in the percentage of lymphocytes and T cells. In addition, her T cells were normally distributed from naïve to memory cells, and her RTEs increased 7 fold, which indicates normalizing of T cell development in the thymus. Furthermore, the percentage of effector memory $CD8^+$ T cells decreased, and a naïve population started appearing. These results demonstrated that donor reconstitution occurred, and that T cell production started. Her NK cell population was still low, but it is expected to increase with time. On the other hand, her B cells were undetectable because the patient was maintained on rituximab since she was still suffering from AIHA after the transplant, and we are waiting to repeat the studies after the effect of rituximab subsides. Interestingly, donor Treg cells were detectable in the patient with normal to elevated expression of Foxp3. In addition, these Tregs are expected to be functional since they express high levels of mediator molecules such as CD25, Helios, and CTLA-4[73,74]. Hence, it is possible that the patient's AIHA might be controlled soon by her own Treg cells, and she could be taken off rituximab.

Expression of IL-7Rα on her donor T cells showed that 44% of her $CD3^+$ T cells expressed IL-7Rα and that they were mostly $CD4^+$ T cells with very few $CD8^+$ T cells that express the IL-7Rα. The reason behind this could be that the transplant regimen did not ablate her own cells and that both donor and recipient T cells are developing simultaneously. In addition, gating only on the IL-$7R\alpha^+$ cells, we determined that the level of expression of IL-7Rα protein on the patient's CD3, CD4, and CD8 cells was around 50% of that of the healthy control cells (Figure 16C). As these cells are of donor origin, this suggests that the family member donor might have been heterozygous for

the same mutation. However, we cannot ascertain this as we did not genotype the donor before the transplant.

In addition, $CD4^+$ T cells isolated from the patient after transplant proliferated better than her $CD8^+$ T cells following stimulation with PHA and anti-CD3 mAbs. To explain, this result correlates with the observed expression of IL-7Rα on the two distinct populations, revealing higher level of expression on the CD4 population, as T cells depend highly on IL-7Rα to divide. These results indicate that the transplant was efficient, but we will continue to assess the reconstitution of the donor HSCs up to a year after transplant, and we expect to see more donor T, B, and NK cells in the periphery with time.

Throughout our study we encountered some limitations that interfered with our planned progress for the research diagnosis:

- Due to the intake of rituximab, we couldn't study the function of the patient's B cells by testing their ability to undergo class switch recombination.
- We stained for the presence of the intracellular soluble IL-7Rα and detected it in the patient's cells similar to the healthy control (data not shown). However, we decided not to show this data because we could not obtain the exact IL-7Rα antibody-binding site on the receptor. Therefore, if the Ab binds to the region of IL-7Rα encoded by exons 1 and 2, it would mean that the truncated form of the protein is expressed and degraded. Alternatively, the Ab used is not specific when used in intracellular staining since it was designed to detect the surface protein. This is why we

did not show these data that will confuse the finding without adding value to the project.

- Another limitation was that we were not sure if the few IL-7Rα positive population of CD3 cells found for the patient are of maternal origin or coming from the expansion of a clone in the periphery. This would require sorting these very few cells and determining their HLA to check for chimerism. Regardless whether they were maternal or self, the T cells being of memory phenotype was a strong clinical indication of SCID as these cells are known to be irresponsive to mitogens showing no proliferative response, which was the case for the patient[75].
- Finally, we were not able to obtain blood samples from the patient on monthly basis to track the success of HSCs engraftment due to COVID-19.

CHAPTER VI

CONCLUSION

Using genetic and functional approaches, we identified a SCID patient with a novel deleterious homozygous mutation in the *IL7Rα* gene and studied the effect of the mutation on protein expression and T cell function. The patient was transplanted at AUBMC so we followed the reconstitution and function of donor cells with time. SCID patients are at a high risk of lethal infections if not diagnosed and treated by HSCT or gene therapy early in life[12]. To our knowledge, this represents the first bedside-to-bench and back to bedside project entirely executed on a patient with SCID at AUB and paves the way for more similar studies in the future.

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