HHS Public Access

Author manuscript

J Clin Immunol. Author manuscript; available in PMC 2021 February 03.

Published in final edited form as:

J Clin Immunol. 2021 January ; 41(1): 136–146. doi:10.1007/s10875-020-00884-6.

Developmental adaptive immune defects associated with STAT5B deficiency in three young siblings

Corinne L. Foley[1,2], **Sareea S. Al Remeithi**[3], **Christopher T. Towe**[4], **Andrew Dauber**[5], **Philippe F Backeljauw**[2], **Leah Tyzinski**[2], **Ashish R. Kumar**[6], **Vivian Hwa**[2]

[1]Immunology Graduate Program, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH, USA

[2]Cincinnati Center for Growth Disorders, Division of Endocrinology, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

[3]Department of Pediatrics, Division of Endocrinology, Sheikh Khalifa Medical City, Abu Dhabi, UAE

[4]Division of Pulmonary Medicine, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

[5]Division of Endocrinology, Children's National Hospital, Department of Pediatrics, George Washington School of Medicine and Health Sciences, Washington, DC, USA

[6]Bone Marrow Transplantation & Immune Deficiency, Cincinnati Children's Hospital Medical Center, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Abstract

Patients with rare homozygous mutations in signal transducer and activator of transcription 5B (STAT5B) develop immunodeficiency resulting in chronic eczema, chronic infections, autoimmunity, and chronic lung disease. STAT5B deficient patients are typically diagnosed in the teenage years, limiting our understanding of the development of associated phenotypic immune abnormalities. We report the first detailed chronological account of post-natal immune dysfunction associated with STAT5B deficiency in humans. Annual immunophenotyping of three siblings

Co-corresponding authors: Ashish Kumar, Ashish.Kumar@cchmc.org, T: 513-803-2994 F: 513-803-1969, Vivian Hwa, Vivian.Hwa@cchmc.org, T: 513-803-7337, F: 513-803-1174.

Authorship Contributions

CF performed experiments, analyzed and interpreted data, and wrote the manuscript. SAR identified the cases and provided clinical data. LT coordinated sample collections and provided clinical data. AD and PB provided endocrine clinical data. CT and AK provided pulmonary and immune clinical data and interpreted data. AK and VH coordinated and supervised the research and manuscript writing. All authors edited the manuscript.

Disclosure of Conflicts of Interest

AK is a consultant for SOBI.

carrying a novel homozygous nonsense mutation in STAT5B was carried out over four years between the ages of 7 months to 8 years. All three siblings demonstrated consistent B cell hyperactivity including elevated IgE levels and autoantibody production, associated with diagnoses of atopy and autoimmunity. Total T cell levels in each sibling remained normal, with regulatory T cells decreasing in the oldest sibling. Interestingly, a skewing toward memory T cells was identified, with the greatest changes in $CD8^+$ effector memory T cells. These results suggest an importance of STAT5B in B cell function and naïve versus memory T cell survival. Progressive dysregulation of $FOXP3^+$ regulatory T cells and $CD8^+$ memory T cell subsets reveal a crucial role of STAT5B in T cell homeostasis. The early diagnosis and focused immune evaluations of these three young STAT5B deficient siblings support an important role of STAT5B in adaptive immune development and function.

Keywords

STAT5B; adaptive immunity; memory T cells; regulatory T cells; development

Introduction

Homozygous recessive mutations in signal transducer and activator of transcription 5B (STAT5B) in humans result in growth hormone insensitivity (GHI) and primary immunodeficiency. STAT5B deficiency (MIM 245590) presents as severe short stature, chronic eczema, autoimmunity, recurrent infections, and chronic pulmonary complications. STAT5B deficient patients experience frequent respiratory infections in infancy and typically develop lymphocytic interstitial pneumonia (LIP) during childhood resulting in fatal respiratory insufficiency before the age of 30 [1]. As patients are typically diagnosed following the development of severe growth and immune impairments, the early diagnosis of STAT5B deficiency can provide valuable insights into how the loss of functional STAT5B drives immune dysfunction and disease development.

STAT5B is one of seven STAT family proteins, sharing 96% amino acid sequence homology with its closest family member, STAT5A. Collectively referred to as Stat5, Stat5a and Stat5b are activated downstream of signaling through type 1 cytokine receptors with ligands including growth hormone (GH) [2–4], IL-2 family cytokines [5–7], IL-3 family cytokines [8], and prolactin [9,10]. Following ligand binding, Janus kinase (JAK) proteins are recruited to the receptor and phosphorylate Stat5, resulting in its dimerization, nuclear translocation, and transcriptional regulation.

In mice, the high homology between Stat5a and Stat5b results in functional redundancy. Only male Stat5b knockout mice have growth impairments while both male and female mice demonstrate mild immune impairments [11–13]. However, both male and female STAT5B deficient patients present with severe short stature and chronic immunodeficiency [1]. When diagnosed clinically, STAT5B deficient patients present with moderate reductions in total $CD3^+$ T cells, naïve $CD4^+$ and $CD8^+$ T cells, $FOXP3^+$ regulatory T cells (Tregs), and occasionally natural killer (NK) cells and TCR$\gamma\delta$ T cells [1,14–16]. These patients also display increased proportions of memory T cells along with B cell hyperactivity including

increased immunoglobulin and autoantibody production [15,17–19]. However, the evolution of immune dysregulation in STAT5B deficiency has not yet been assessed early in life.

We now report a novel homozygous loss-of-function (LOF) STAT5B mutation in three siblings initially presenting between ages 7 months to 5 years. The early identification of a pathological molecular defect by whole exome sequencing (WES) allowed for early diagnosis and longitudinal evaluation of each sibling. We tracked variations in adaptive immune cell subsets and disease development through infancy and early childhood. Our findings suggest important roles for STAT5B in the maintenance of T cell subsets including $FOXP3^+$ Tregs and $CD8^+$ memory T cells. Furthermore, sustained impairments in B cells and in the proportions of naïve versus memory T cells were identified, suggesting a role of STAT5B in immune tolerance and T cell homeostasis. Collectively, our analyses highlight the importance of STAT5B in the development and maintenance of adaptive immune cells.

Materials and Methods

Antibodies

Antibodies used were: anti-phospho-STAT5 and anti-alpha tubulin monoclonal horseradish peroxidase conjugate (Cell Signaling Technology, Danvers, MA); anti-FLAG monoclonal antibody (Sigma-Aldrich Corp., St. Louis, MO); following recommended dilutions. Secondary antibodies (horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG antibodies) were obtained from Amersham Biosciences (Uppsala, Sweden) and fluorescently tagged antibodies for microscopy were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Thermo Scientific (Waltham, MA).

Mutation Analysis

Whole exome sequencing of DNA from Patient 1, P1, was performed through Sengenics (Cambridge, England) on Illumina HiSeq 2500. Sanger sequencing confirmed the homozygous mutation in P1 and revealed heterozygosity in both parents. Dermal fibroblasts established from skin biopsies from each sibling were obtained with consent in compliance with ethics guidelines (institutional review board of Cincinnati Children’s Hospital Medical Center), and DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). For confirming the mutation, sanger sequencing of STAT5B exon 15 was performed using primers; (forward) 5’-ctctggtgttcttatgttcacttgttgtatct-3’ (reverse) 5’-tatttttcataggctgccttattatgagtatgtca-3’.

Serum Assays

Serum samples from the patients were obtained with consent and in compliance with the institutional review board of Cincinnati Children’s Hospital Medical Center. Serum GH, IGF-1, IGF-BP3, and IGF-ALS (acid labile subunit) were clinically measured using chemiluminescent immunoassay. Serum prolactin was measured using carbonylmetalloimmunoassay. The leukocyte count was determined through flow cytometry, fluorescence, and absorption spectrophotometry. The remaining immune profiles were obtained through flow cytometry. All laboratory tests were performed at Cincinnati Children’s Hospital Medical Center.

Generation of Recombinant Mutant

For reconstitution experiments, the STAT5B p.W631* was generated using the Q5 Site-Directed Mutagenesis Kit from New England BioLabs (#E0554S), with N-terminally FLAG-tagged STAT5B cDNA as template [(20)]. The primers were: (forward) 5'-gcatcaccattgcttagaagtttgattctcagg-3' and (reverse) 5'-cctgagaatcaaacttctaagcaatggtgatgc-3'. The mutated nucleotide is underlined, and mutant cDNA sequence was confirmed by Sanger sequencing.

Cell Culture

HEK293 cells stably transfected with the human growth hormone receptor (HEK-GHRs) provided by Dr. Richard J. Ross (University of Sheffield, Sheffield, UK) [(21)] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 0.4% Geneticin at 37°C in 5% CO_2. For *in vitro* functional analyses, HEK293-GHRs were transfected with either an empty pcDNA 3.1 vector, FLAG-tagged STAT5B WT (F-STAT5B WT), or FLAG-tagged STAT5B mutant (F-STAT5B p.W631*) using Polyjet transfection reagent (SignaGen Laboratories). Following a 24 hour transfection, cells were serum starved in DMEM supplemented with 0.1% bovine serum albumin (BSA) for 16 hours and then treated with 100 ng/mL rhGH for 20 minutes [(22)]. Transfection experiments were repeated three times.

Western Immunoblotting

Cells were solubilized in Sigma M2 Cell Lysis Buffer (pH 7.5, 50 mM tris(hydroxymethyl)aminomethane (Tris), 150nM sodium chloride, ethylenediaminetetraacetic acid (EDTA), 1% Triton-X100) supplemented with a protease inhibitor tablet (Roche Applied Science) and 100 mM sodium orthovanadate. Protein concentrates of the lysates were determined using the Bradford assay, and 25 μg protein was loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (SDS-PAGE). The gel-fractionated proteins were then transferred onto nitrocellulose membranes and blocked using 3% BSA in 1X Tris-buffered saline. Western blots were processed with the appropriate primary and secondary antibodies following the manufacturers' protocols, and scanned using the *Odyssey IR Imaging System* (LICOR Biosciences).

Immunocytochemistry

HEK-GHRs were plated at a density of 25,000 cells per well in 8-well chamber slides, transfected and treated as outlined above, and fixed with 100% methanol for 5 minutes at −20°C. After 4 hours of blocking in 5% donkey serum, the wells were incubated overnight in the appropriate primary and secondary antibodies according to the manufacturers' protocols. Images were captured using a Nikon Ti-E inverted microscope with A1R confocal, GaAsp PMTs, and resonant scanner. Images were analyzed using the Imaris software and statistical analyses performed using GraphPad Prism. To calculate significant differences, the Holm-Sidak method was used with alpha=0.5.

Results

Clinical presentation of three siblings

Three siblings, born to consanguineous parents, were referred to the endocrine outpatient clinic at Sheikh Khalifa Medical City in Abu Dhabi for severe post-natal growth failure. The oldest sibling, patient 1 (P1), was conceived by in-vitro fertilization and born premature at 25 weeks but appropriate for gestational age (AGA) with a birth weight of 650-g and birth length of 30 cm. As a neonate, P1 was hospitalized for pneumonia with sepsis, requiring antibiotic treatments and supplemental oxygen. After discharge, she developed recurrent urinary tract and pulmonary infections, resulting in multiple hospitalizations that reduced in frequency with age and responded to antibiotics. She additionally had severe bronchopulmonary dysplasia, feeding difficulties resulting in failure to thrive, and was developmentally delayed. P1 presented to Cincinnati Children's Hospital Medical Center (CCHMC) at 4.8 years old with a GHI phenotype including severe short stature (height SDS −6.5), centripetal fat distribution, a prominent forehead, and a depressed nasal bridge. Atopic disease was present including eczema and reactive airway disease requiring corticosteroids. P1 was diagnosed with autoimmune thyroiditis and treated with levothyroxine. She additionally developed idiopathic thrombocytopenia at age 4 that resolved in months and recurred at age 7. She showed a temporarily positive treatment response to IVIG and dexamethasone, but platelet counts were normalized and sustained upon treatment with sirolimus. She did not develop ear, sinus, or skin infections or fungal infections. No lymphadenopathy was noted on physical exams.

Patient 2 (P2) was born AGA at 37 weeks, weighing 2040-g. At the time of presentation, he was 2 years old with severe short stature and height SDS of −5.06, displaying a GHI phenotype similar to P1. P2 developed recurrent infections of the skin, urinary tract, and lungs requiring antibiotics and often supplemental oxygen. He presented with general lymphadenopathy at the time of evaluation. Similar to P1, chronic eczema and reactive airway disease developed. At presentation, P2 was diagnosed with autoimmune thyroiditis and treated with levothyroxine.

Patient 3 (P3) presented at 7 months of age and was born AGA at 35 weeks, weighing 1900-g, but presented with severe short stature (height SDS −4.7) and facial dysmorphism. She had failure to thrive, and experienced recurrent skin and pulmonary infections requiring antibiotics and supplemental oxygen. P3 initially presented with atopic eczema and over the years of follow-up developed reactive airway disease. At age 2, she was diagnosed with autoimmune thyroiditis and treated with levothyroxine. P3 additionally had gastroesophageal reflux disease. No lymphadenopathy was noted.

Family history was negative for severe short stature that required medical attention, and both parents were of near-normal heights. When evaluated at CCHMC, the three siblings were placed on recombinant human insulin-like growth factor 1 (rhIGF-1) to improve growth. The clinical presentation of these three siblings is outlined in Table 1.

Identification of novel homozygous nonsense STAT5B mutation in three siblings

At presentation, each sibling had normal serum GH levels and below-normal IGF-1, IGF-BP3, and acid labile subunit (ALS) concentrations, consistent with GHI. These abnormally low values and clinical phenotype prompted WES analysis of P1 at age 3.9. Trio clinical whole exome sequencing revealed 112,781 variants in P1, with only one candidate associated with growth and immune impairments, a homozygous variant in *STAT5B*, c.1892G>A, converting a tryptophan at position 631 to a nonsense residue, p.W631*. Other candidate variants can be found in Table S2. Sanger sequencing revealed heterozygosity in this STAT5B locus in both parents (Figure S1A), and sequencing of dermal fibroblast cultures from each sibling confirmed the presence of the mutation (Figure S1B). Interestingly, this novel mutation is adjacent to the first identified mutation in STAT5B, p.A630P [23] (Figure S1C). The resulting protein lacks ~50% of the Src Homology 2 (SH2) domain, the critical phosphorylated tyrosine at position 699, and the entire transactivation domain (TAD).

Reconstitution analyses reveal impairments in the expression and function of the STAT5B p.W631* protein

The SH2 and TAD domains are critical for STAT5B activation, homodimer formation, and driving transcriptional activation [24]. To evaluate the functional capacity of the STAT5B p.W631* mutation, we performed *in vitro* reconstitution analyses in HEK-GHRs. The truncated STAT5B p.W631* protein was significantly reduced in expression compared to wild-type FLAG-STAT5B (F-STAT5B WT) (Figure 1A *and* B). F-STAT5B WT, but not F-STAT5B p.W631*, was phosphorylated following GH treatment (Figure 1A). Immunofluorescence analyses revealed impaired nuclear localization of the F-STAT5B p.W631* protein following GH treatment (Figure 1C *and* D). Our results reveal reduced expression of the STAT5B p.W631* variant and an inability to be phosphorylated or traffic into the nucleus upon GH stimulation.

Sustained B cell hyperactivity is associated with atopy and autoimmunity in STAT5B deficient siblings

The identification of a LOF STAT5B variant in our patients prompted an evaluation of immune profiles. STAT5B deficient patients typically have reductions in the number of NK cells along with impaired function, and normal to elevated levels of B cells with increased immunoglobulin production [1,15,17,19,25–28]. From infancy through early childhood, each sibling displayed normal numbers of NK cells (Table S1), B cells, and serum levels of IgG, IgA, and IgM (Table 2). Serum levels of IgE were consistently elevated in P1 and P2 (Table 2). These sustained elevations in IgE were associated with the development of chronic eczema and reactive airway disease, consistent with an atopic phenotype often associated with STAT5B deficiency [1,15,17]. In early childhood, each sibling carried anti-thyroglobulin and anti-thyroid peroxidase antibodies associated with autoimmune thyroiditis (Table 2).

STAT5B is important for the maintenance of T cell levels

While immune defects associated with LOF STAT5B mutations vary [26], 7 out of 9 STAT5B deficient patients evaluated were moderately to severely T cell lymphopenic at the time of diagnosis. It remains unknown whether the systemic T cell reduction in these patients is due to impaired thymic T cell production or peripheral destruction. Studies of *Stat5b*$^{-/-}$ mice revealed normal levels of thymocytes and reduced splenocytes [11,29], while *Stat5a*$^{-/-}$*b*$^{-/-}$ mice showed significant reductions in both thymocytes and splenocytes [30]. To evaluate thymic output of T cells in the absence of functional STAT5B, levels of recent thymic emigrants (RTE) were assessed by quantification of CD4^{+}CD45^{+}CD31^{+} T cells. At 7 months of age, P3 displayed reduced RTEs that normalized the following year (Table 3). At 2 years of age, P2 displayed a low-normal RTE level that normalized over the following years. P1, who was evaluated at 4 years old, had normal RTEs throughout the evaluation.

Annual immune analyses did not reveal any alterations in total T cell levels, with the absolute number and total percentage of CD3^{+}, CD4^{+}, and CD8^{+} T cells remaining within normal ranges in all three siblings (Table 3). The CD4:CD8 ratio also remained normal as previously reported [25]. The normal levels of peripheral T cells from infancy to early childhood in the three young siblings suggest that STAT5B is likely dispensable for T lymphocyte development in these siblings, but may be important for long-term maintenance of T cell levels as older STAT5B deficient patients typically manifest T cell lymphopenia [26].

STAT5B may be responsible for the maintenance, rather than generation, of regulatory T cells

Roles of STAT5B in IL-2 signaling include FOXP3 induction, Treg function, and self-tolerance in both humans and mice [18,31–34]. Prior reports demonstrate reduced numbers and function of Tregs in STAT5B deficient patients [32]; however, these evaluations were made when the patients were older and had already developed severe immune and pulmonary complications. In our younger patients, we annually evaluated the absolute numbers and total percentage of Tregs identified as CD4^{+} cells either expressing high levels of CD25 and low levels of CD127 (CD25^{+}CD127^{low}) or CD25 and FOXP3 (CD25^{+}FOXP3^{+}). Interestingly, the absolute number and total percentage of FOXP3+ Tregs were normal in P2 and P3 between the ages of 7 months and 5 years (Figure 2A–B, Figure S2). Reductions in Tregs were not consistently identified in P1 until age 8 (Figure 2A–C). However, the total percentage of CD4^{+}CD25^{+}CD127^{low} T cells was reduced by the age of 3 years in each sibling (Figure 2D). These data suggest that the LOF STAT5B variant in these siblings impairs the maintenance of FOXP3 expression over time rather than the initial generation of FOXP3^{+} Tregs.

Pronounced dysregulation of memory CD8^{+} T cells in young STAT5B deficient siblings

When evaluated, STAT5B deficient patients often have reduced naïve T cells and elevations in T cells with a memory/activation phenotype [15,19,25]. Each of our siblings had modest but persistently reduced proportions of both CD4^{+} and CD8^{+} naïve T cells and elevated proportions of CD4^{+} and CD8^{+} memory T cells (Figure S3, Figure S4A *and* B, Figure 3A *and* B). Evaluation of CD4^{+} T cell activation markers including CD69^{+}, CD71^{+}, CD40L^{+},

CD134$^+$, HLA$^+$, and CD95$^+$, were unremarkable (Table S1). The impact of STAT5B on memory T cell subsets including CD45RO$^+$CCR7$^+$ central memory (TCM), CD45RO$^+$CCR7$^-$ effector memory (TEM), and CD45RA$^+$CCR7$^-$ effector memory cells re-expressing CD45RA (TEMRA) were also evaluated in each sibling. CD4$^+$ and CD8$^+$ TCM cells remained within normal ranges (Figure S4C, Figure 3C). No alterations in the proportion CD4$^+$ TEM cells were identified (Figure S4D), but increases in CD8$^+$ TEM cell proportions became clinically significant with age in each sibling (Figure 3D). Elevations in the percentage of CD8+ TEMRA cells detected during infancy in P2 and P3 normalized around age 4 and remained normal in P1 (Figure 3E). A dysregulation of CD8$^+$ T cells was noted despite normal CD4$^+$ memory T cell subsets, an observation not previously reported for STAT5B deficiency. Our patients carried elevated proportions of CD8+ memory T cells beginning in infancy, suggesting a critical role for this LOF STAT5B variant in CD8$^+$ T cell activation and homeostasis.

Discussion

Homozygous mutations in STAT5B occur rarely in humans and are typically diagnosed in the teenage years following the development of prominent growth and immune impairments. The late diagnosis of this condition, together with a discordance of phenotypic similarities between *Stat5*$^{-/-}$ mice, have limited our understanding of the role of STAT5B in the development of adaptive immune dysfunction in humans. The four-year evaluation of three STAT5B deficient siblings, from ages 7 months to 8 years, permitted the generation of a potential timeline of immune dysregulation and disease development associated with STAT5B deficiency (Figure 4).

At presentation, our STAT5B deficient siblings exhibited severe short stature, atopic eczema, autoimmune thyroiditis, and chronic infections. The pneumonias often required supplemental oxygen, but hospitalizations due to pulmonary complications decreased with age. Lymphocytic interstitial pneumonia, the fatal sequelae of STAT5B deficiency, has not yet presented in these siblings. Sequencing identified a novel homozygous nonsense mutation in the SH2 domain of STAT5B, STAT5B p.W631*, resulting in a truncated STAT5B variant with reduced expression and an inability to be phosphorylated and translocate to the nucleus upon stimulation.

As these siblings were genetically diagnosed with STAT5B deficiency at an early age and had not yet developed severe lung complications, we had the unique opportunity to evaluate developmental patterns of adaptive immune cells due to a lack of functional STAT5B. The young age and severe short stature of these patients prevented the collection of samples for mechanistic analyses. However, novel insights were gained from focused clinical adaptive immune evaluations. While normal levels of B cells were noted, we identified elevated IgE production and autoantibody production associated with atopy and autoimmunity in the first year of life. At their young ages, no T cell lymphopenia was noted, and a reduction in FOXP3+ Tregs was not identified until age 8 in P1. While a reduction in the proportion of naïve T cells and elevation in the proportion of memory T cells was sustained through the years of analysis, CD8$^+$ T cells had more pronounced impairments with an increase in CD8$^+$ TEM cells and a reduction of CD8$^+$ TEMRA cells with age. Our annual evaluations of three

young siblings carrying a LOF STAT5B variant highlight the importance of STAT5B in post-natal immune development and function.

Loss of immune regulation was evident in infancy in all three siblings resulting in sustained IgE overproduction and autoantibody production. These effects were associated with the development of atopy and autoimmunity during infancy, suggesting a role for STAT5B in immune tolerance. Mouse studies have determined a critical role for the loss of Stat5 in T follicular helper (Tfh) cell differentiation, along with increased germinal center B cells and autoantibody production [35,36]. Future evaluations of Tfh cells may help to reveal a mechanistic role for B cell hyperactivity associated with STAT5B deficiency as evidenced from other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis [37].

Moderate to severe T cell lymphopenia is often associated with STAT5B deficiency [19,26]. We show that T cell lymphopenia may likely be developmental result of a loss of STAT5B in our young siblings. From 7 months to 8 years of age, all three siblings had normal levels of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. It is possible that the truncated variant of STAT5B p.W631* retains some functional capacity, allowing for normal T cell levels. However, as this variant lacks critical functional domains and demonstrated impaired activation and nuclear translocation in response to stimulation, it is less likely that this truncated variant remains functional. Our clinical evaluations suggest an involvement of STAT5B in the maintenance of $CD3^+$ T cells rather than their generation. As Stat5 in mice is known to induce expression of anti-apoptotic proteins [30,31,38,39], the evaluation of apoptotic markers in T cells derived from STAT5B deficient patients may be insightful.

Tregs have been extensively characterized in STAT5B deficient patients including reports of reductions in thymic and induced Tregs and identifying the binding sites of STAT5B on the FOXP3 promoter region [18,31,32]. Signaling through STAT5B induces expression of CD25 and FOXP3, but whether this is essential from birth remained unknown. Our observations suggest a potential role for STAT5B in the maintenance of regulatory T cells in our three siblings, as no reductions in $FOXP3^+$ Tregs were observed until later in childhood. Mechanisms other than STAT5B signaling that regulate FOXP3 expression and regulatory T cell function including TGF-β [40] and retinoic acid signaling [41], may be sufficient at early ages to generate and maintain Tregs. We show for the first time that a LOF mutation in STAT5B can result in impaired human Treg homeostasis with age.

When evaluated, older STAT5B deficient patients carry elevated $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ memory T cells and reduced $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ naïve T cells [15,19,25]. This was similarly observed in our younger patients; however, $CD4^+$ memory T cell subsets remained within normal ranges. As the first evaluation of these cell types in STAT5B deficiency, $CD8^+$ TEM and TEMRA cells in our patients changed significantly over time. As $CD8^+$ TEM levels increased above the normal range, $CD8^+$ TEMRA cells decreased to within normal range. While most functional assays on patient-derived STAT5B deficient T cells have been performed on $CD4^+$ T cells, mouse studies have identified significant differences in $CD8^+$ T cells with alterations in Stat5 expression [29,36,39]. Although signaling through IL-7 and IL-15 is known to be involved in murine

memory T cell development [42,43], the specific signaling components involved in human memory T cell development remain unknown. Similarly, the mechanisms governing human TCM versus TEM development remain elusive [42,44]. Our analyses suggest that STAT5B plays a critical role in the generation or survival of memory T cells including $CD8^+$ TEM cells in our siblings, although the specific mechanisms involved still need to be determined. As a downstream transcription factor involved in IL-7 and IL-15 signaling, STAT5B activation may be important for the regulation of homeostatic proliferation and survival of CD8+ memory T cells. Interestingly, mouse studies have concluded that Stat5 activation is sufficient for $CD8^+$ memory T cell survival [29,45,46], while the loss of STAT5B in humans may support memory T cell survival [15,19,25].

With the lack of innate immune impairments in our young patients, we focused on evaluating adaptive immune cell subsets. Mechanistic assessments of these progressive changes were unable to be performed due to insufficient samples collected, given the size and young age of these patients. Nevertheless, our results are the first to suggest a critical role for STAT5B in T cell development from infancy to early childhood, highlighting the importance of early diagnosis and evaluations of patients presenting with developmental delay, postnatal growth impairments, eczema, chronic infections, and autoimmunity. As LIP has not yet developed in these young siblings, along with T cell lymphopenia and significantly reduced regulatory T cell levels, we will continue to closely monitor these markers and their potential involvement in STAT5B-associated lung disease development. Total and regulatory T cell values should be carefully evaluated following the diagnosis of STAT5B deficiency, along with symptoms of pulmonary disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the patients and family for their collaboration and participation, and the diagnostic immunology laboratory at CCHMC for providing raw flow cytometry data. We also thank David Hildeman for his assistance in reviewing the manuscript.

References

1. Hwa V, Nadeau K, Wit JM, Rosenfeld RG. STAT5b deficiency: Lessons from STAT5b gene mutations. Best Pract Res Clin Endocrinol Metab. 2011 2;25(1):61–75. [PubMed: 21396575]
2. Gouilleux F, Wakao H, Mundt M, Groner B. Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J. 1994 9 15;13(18):4361–9. [PubMed: 7925280]
3. Waxman DJ, Ram PA, Park SH, Choi HK. Intermittent plasma growth hormone triggers tyrosine phosphorylation and nuclear translocation of a liver-expressed, Stat 5-related DNA binding protein. Proposed role as an intracellular regulator of male-specific liver gene transcription. J Biol Chem. 1995 6 2;270(22):13262–70. [PubMed: 7768925]
4. Wood TJ, Sliva D, Lobie PE, Pircher TJ, Gouilleux F, Wakao H, et al. Mediation of growth hormone-dependent transcriptional activation by mammary gland factor/Stat 5. J Biol Chem. 1995 4 21;270(16):9448–53. [PubMed: 7721871]

5. Hou J, Schindler U, Henzel WJ, Wong SC, McKnight SL. Identification and purification of human Stat proteins activated in response to interleukin-2. Immunity. 1995 4;2(4):321–9. [PubMed: 7719937]

6. Lin J-X, Migone T-S, Tseng M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity. 1995 4 1;2(4):331–9. [PubMed: 7719938]

7. Wakao H, Harada N, Kitamura T, Mui AL, Miyajima A. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J. 1995 6 1;14(11):2527–35. [PubMed: 7781605]

8. Mui AL, Wakao H, O'Farrell AM, Harada N, Miyajima A. Interleukin-3, granulocytemacrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. EMBO J. 1995 3 15;14(6):1166–75. [PubMed: 7720707]

9. Schmitt-Ney M, Doppler W, Ball RK, Groner B. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. Mol Cell Biol. 1991 7 1;11(7):3745–55. [PubMed: 2046676]

10. Wakao H, Gouilleux F, Groner B. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J. 1994 5 1;13(9):2182–91. [PubMed: 7514531]

11. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, et al. Stat5b Is Essential for Natural Killer Cell–mediated Proliferation and Cytolytic Activity. J Exp Med. 1998 12 7;188(11):2067–74. [PubMed: 9841920]

12. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park S-H, Ram PA, et al. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci U S A. 1997 7 8;94(14):7239–44. [PubMed: 9207075]

13. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, et al. Stat5a and Stat5b Proteins Have Essential and Nonessential, or Redundant, Roles in Cytokine Responses. Cell. 1998 5 29;93(5):841–50. [PubMed: 9630227]

14. Hwa V, Camacho-Hübner C, Little BM, David A, Metherell LA, El-Khatib N, et al. Growth Hormone Insensitivity and Severe Short Stature in Siblings: A Novel Mutation at the Exon 13-Intron 13 Junction of the <i>STAT5b</i> Gene. Horm Res Paediatr. 2007 3 28;68(5):218–24.

15. Bezrodnik L, Giovanni DD, Caldirola MS, Azcoiti ME, Torgerson T, Gaillard MI. Long-Term Follow-up of STAT5B Deficiency in Three Argentinian Patients: Clinical and Immunological Features. J Clin Immunol. 2015 4 1;35(3):264–72. [PubMed: 25753012]

16. Pugliese-Pires PN, Tonelli CA, Dora JM, Silva PCA, Czepielewski M, Simoni G, et al. A novel STAT5B mutation causing GH insensitivity syndrome associated with hyperprolactinemia and immune dysfunction in two male siblings. Eur J Endocrinol. 2010 8 1;163(2):349–55. [PubMed: 20538865]

17. Nadeau K, Hwa V, Rosenfeld RG. STAT5b Deficiency: An Unsuspected Cause of Growth Failure, Immunodeficiency, and Severe Pulmonary Disease. J Pediatr. 2011 5;158(5):701–8. [PubMed: 21414633]

18. Kanai T, Jenks J, Nadeau KC. The STAT5b Pathway Defect and Autoimmunity. Front Immunol [Internet]. 2012 8 14 [cited 2016 Jul 13];3 Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3418548/

19. Acres MJ, Gothe F, Grainger A, Skelton AJ, Swan DJ, Willet JDP, et al. STAT5B deficiency due to a novel missense mutation in the coiled-coil domain. J Allergy Clin Immunol [Internet]. 2018 9 8 [cited 2018 Sep 13]; Available from: http://www.sciencedirect.com/science/article/pii/S0091674918312806

20. Hwa V, Little B, Kofoed EM, Rosenfeld RG. Transcriptional regulation of insulin-like growth factor-I by interferon-gamma requires STAT-5b. J Biol Chem. 2004 1 23;279(4):2728–36. [PubMed: 14570891]

21. Maamra M, Finidori J, Laue SV, Simon S, Justice S, Webster J, et al. Studies with a Growth Hormone Antagonist and Dual-fluorescent Confocal Microscopy Demonstrate that the Full-length Human Growth Hormone Receptor, but Not the Truncated Isoform, Is Very Rapidly Internalized

Independent of Jak2-Stat5 Signaling. J Biol Chem. 1999 5 21;274(21):14791–8. [PubMed: 10329677]

22. Klammt J, Neumann D, Gevers EF, Andrew SF, Schwartz ID, Rockstroh D, et al. Dominant-negative STAT5B mutations cause growth hormone insensitivity with short stature and mild immune dysregulation. Nat Commun [Internet]. 2018 12 [cited 2019 Feb 20];9(1). Available from: http://www.nature.com/articles/s41467-018-04521-0
23. Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, Tsubaki J, et al. Growth Hormone Insensitivity Associated with a STAT5b Mutation. N Engl J Med. 2003 9 18;349(12):1139–47. [PubMed: 13679528]
24. Levy DE, Darnell JE. STATs: transcriptional control and biological impact. Nat Rev Mol Cell Biol. 2002 9;3(9):651–62. [PubMed: 12209125]
25. Bernasconi A, Marino R, Ribas A, Rossi J, Ciaccio M, Oleastro M, et al. Characterization of Immunodeficiency in a Patient With Growth Hormone Insensitivity Secondary to a Novel STAT5b Gene Mutation. Pediatrics. 2006 11 1;118(5):e1584–92. [PubMed: 17030597]
26. Hwa V STAT5B deficiency: Impacts on human growth and immunity. Growth Horm IGF Res. 2016 6;28:16–20. [PubMed: 26703237]
27. Hwa V, Little B, Adiyaman P, Kofoed EM, Pratt KL, Ocal G, et al. Severe Growth Hormone Insensitivity Resulting from Total Absence of Signal Transducer and Activator of Transcription 5b. J Clin Endocrinol Metab. 2005 7 1;90(7):4260–6. [PubMed: 15827093]
28. Vargas-Hernández A, Witalisz-Siepracka A, Prchal-Murphy M, Klein K, Mahapatra S, Al-Herz W, et al. Human signal transducer and activator of transcription 5b (STAT5b) mutation causes dysregulated human natural killer cell maturation and impaired lytic function. J Allergy Clin Immunol. 2020 1 1;145(1):345–357.e9. [PubMed: 31600547]
29. Kelly J, Spolski R, Imada K, Bollenbacher J, Lee S, Leonard WJ. A Role for Stat5 in CD8+ T Cell Homeostasis. J Immunol. 2003 1 1;170(1):210–7. [PubMed: 12496402]
30. Yao Z, Cui Y, Watford WT, Bream JH, Yamaoka K, Hissong BD, et al. Stat5a/b are essential for normal lymphoid development and differentiation. Proc Natl Acad Sci U S A. 2006 1 24;103(4):1000–5. [PubMed: 16418296]
31. Kanai T, Seki S, Jenks JA, Kohli A, Kawli T, Martin DP, et al. Identification of STAT5A and STAT5B Target Genes in Human T Cells. PLoS ONE [Internet]. 2014 1 30 [cited 2016 Jul 18];9(1). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3907443/
32. Jenks JA, Seki S, Kanai T, Huang J, Morgan AA, Scalco RC, et al. Differentiating the roles of STAT5B and STAT5A in human CD4+ T cells. Clin Immunol Orlando Fla. 2013 8;148(2):227–36.
33. Antov A, Yang L, Vig M, Baltimore D, Parijs LV. Essential Role for STAT5 Signaling in CD25+CD4+ Regulatory T Cell Homeostasis and the Maintenance of Self-Tolerance. J Immunol. 2003 10 1;171(7):3435–41. [PubMed: 14500638]
34. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, et al. Nonredundant roles for Stat5a/b in directly regulating Foxp3. Blood. 2007 5 15;109(10):4368–75. [PubMed: 17227828]
35. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S. STAT5 is a potent negative regulator of TFH cell differentiation. J Exp Med. 2012 2 13;209(2):243–50. [PubMed: 22271576]
36. Villarino A, Laurence A, Robinson GW, Bonelli M, Dema B, Afzali B, et al. Signal transducer and activator of transcription 5 (STAT5) paralog dose governs T cell effector and regulatory functions. eLife [Internet]. 5 Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4856466/
37. Kurata I, Matsumoto I, Sumida T. T follicular helper cell subsets: a potential key player in autoimmunity. Immunol Med. 2020 6 17;0(0):1–9.
38. Behbod F, Nagy ZS, Stepkowski SM, Karras J, Johnson CR, Jarvis WD, et al. Specific Inhibition of Stat5a/b Promotes Apoptosis of IL-2-Responsive Primary and Tumor-Derived Lymphoid Cells. J Immunol. 2003 10 15;171(8):3919–27. [PubMed: 14530308]
39. Tripathi P, Kurtulus S, Wojciechowski S, Sholl A, Hoebe K, Morris SC, et al. STAT5 Is Critical To Maintain Effector CD8+ T Cell Responses. J Immunol. 2010 8 15;185(4):2116–24. [PubMed: 20644163]
40. Xu L, Kitani A, Strober W. Molecular mechanisms regulating TGF-β-induced Foxp3 expression. Mucosal Immunol [Internet]. 2010 3 10 [cited 2017 Jun 16]; Available from: http://www.nature.com/mi/journal/vaop/ncurrent/full/mi20107a.html

41. Gao Y, Lin F, Su J, Gao Z, Li Y, Yang J, et al. Molecular mechanisms underlying the regulation and functional plasticity of FOXP3+ regulatory T cells. Genes Immun. 2012 1;13(1):1–13. [PubMed: 22048454]

42. Boyman O, Létourneau S, Krieg C, Sprent J. Homeostatic proliferation and survival of naïve and memory T cells. Eur J Immunol. 2009;39(8):2088–94. [PubMed: 19637200]

43. Sprent J, Surh CD. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nat Immunol. 2011 6;12(6):478–84. [PubMed: 21739670]

44. Willinger T, Freeman T, Hasegawa H, McMichael AJ, Callan MFC. Molecular Signatures Distinguish Human Central Memory from Effector Memory CD8 T Cell Subsets. J Immunol. 2005 11 1;175(9):5895–903. [PubMed: 16237082]

45. Hand TW, Cui W, Jung YW, Sefik E, Joshi NS, Chandele A, et al. Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. Proc Natl Acad Sci. 2010 9 21;107(38):16601–6. [PubMed: 20823247]

46. Grange M, Giordano M, Mas A, Roncagalli R, Firaguay G, Nunes JA, et al. Control of CD8 T cell proliferation and terminal differentiation by active STAT5 and CDKN2A/CDKN2B. Immunology. 2015 8;145(4):543–57. [PubMed: 25882552]

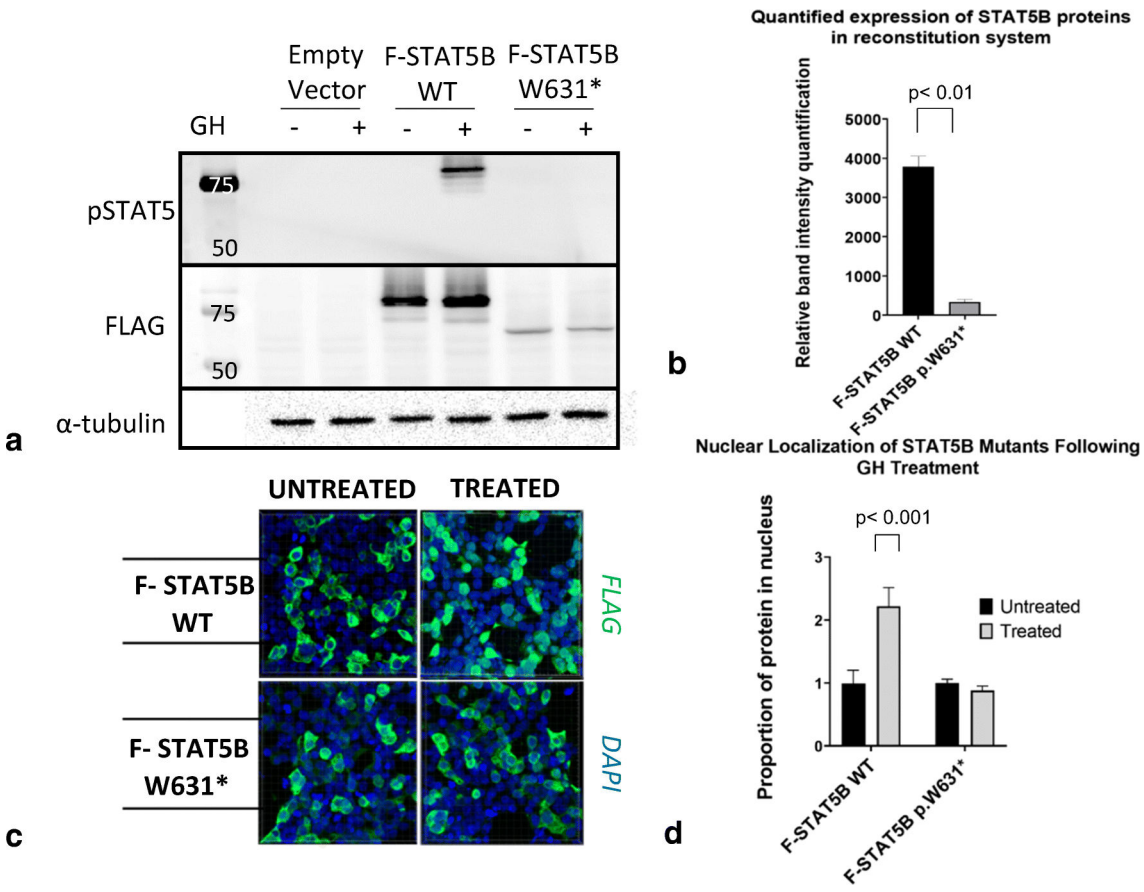

Figure 1: Functional impairments of the STAT5B W631* protein

HEK293 reconstitution analyses of wild type F-STAT5B and mutant F-STAT5B-W631* in response to treatment with 100ng/mL rhGH. (A) Representative immunoblot analysis from three repeats; (B) densitometric quantification of F-STAT5B protein expression from immunoblot; (C) cellular localization by triplicate immunofluorescence analysis. FLAG, green; nucleus, blue; (D) quantification of nuclear localization.

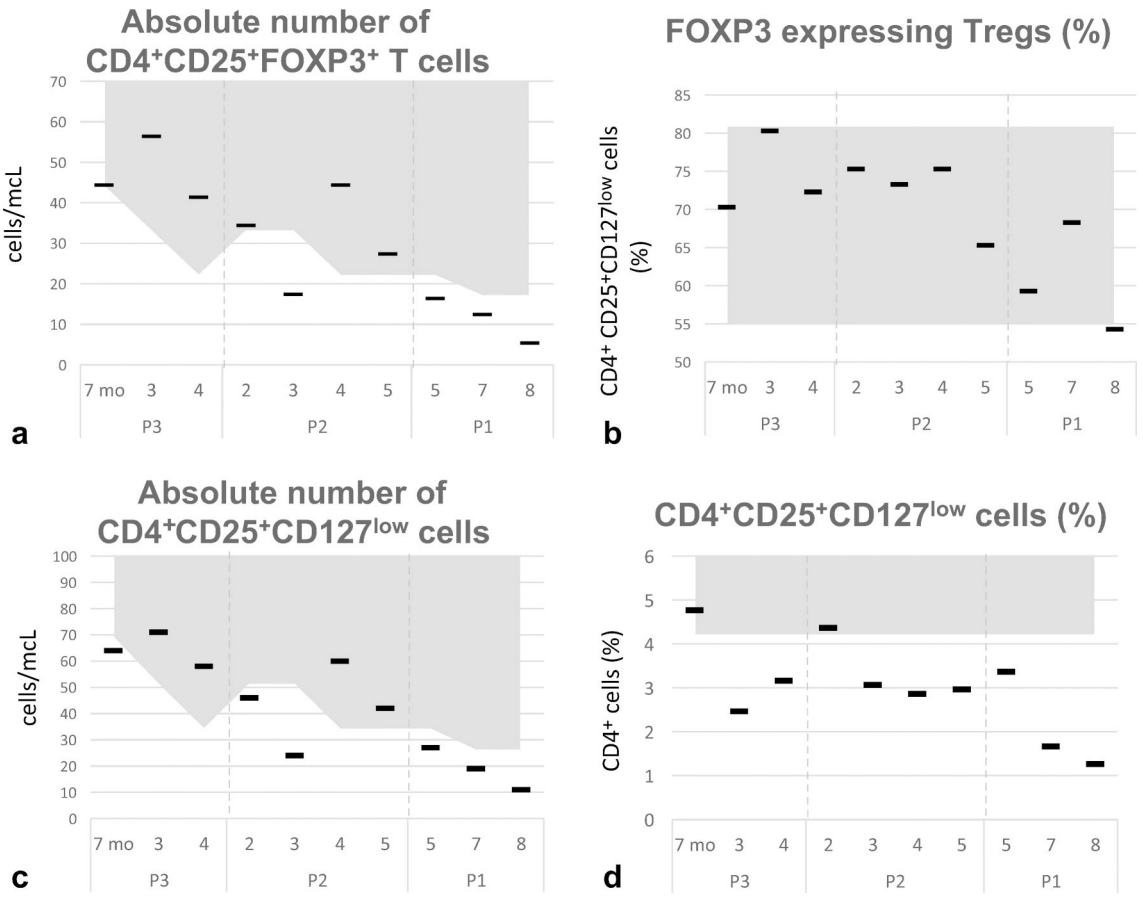

Figure 2: Regulatory T cell values over time in three STAT5B deficient siblings.

(A-B) Absolute number and total percentage of $CD4^+CD25^+FOXP3^+$ Tregs over time in three siblings. (C-D) Absolute number and total percentage of $CD4^+CD25^+CD127^{low}$ Tregs over time in three siblings. Grey area indicates normal range of T cell values. X-axis, age (years) of analysis for each proband.

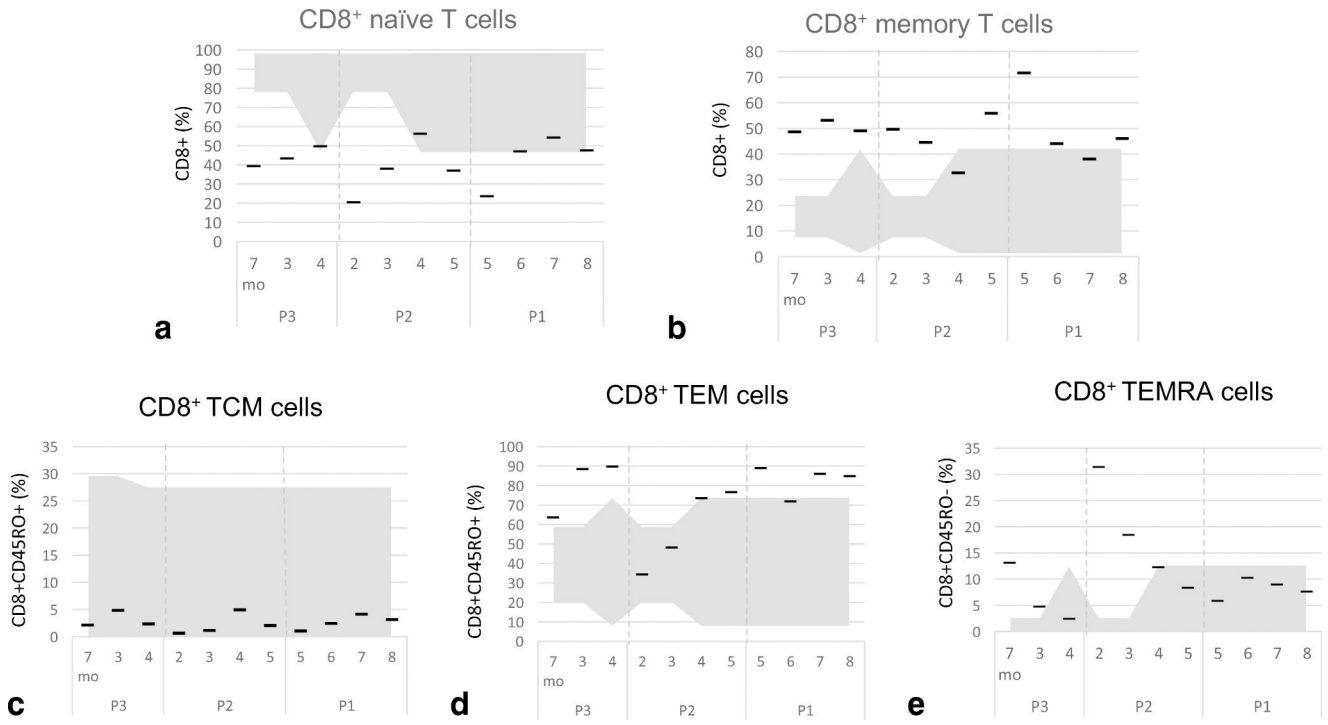

Figure 3: CD8+ T cell dysregulation in STAT5B deficient siblings over time.

(A) Percentage of $CD8^+$ $CD45RA^+$ (naïve) T cells. (B) Percentage of $CD8^+CD45RO^+$ (memory) T cells. (C-E) Percentage of memory T cell subsets including (C) $CD8^+CD45RO^+CCR7^+$ central memory T cells, (E) $CD8^+CD45RO^+CCR7^-$ effector memory T cells, and (F) $CD8^+CD45RA^+CCR7^-$ CD45RA re-expressing effector memory T cells. Grey area indicates normal range of T cell values. X-axis, age (years) of analysis for each proband.

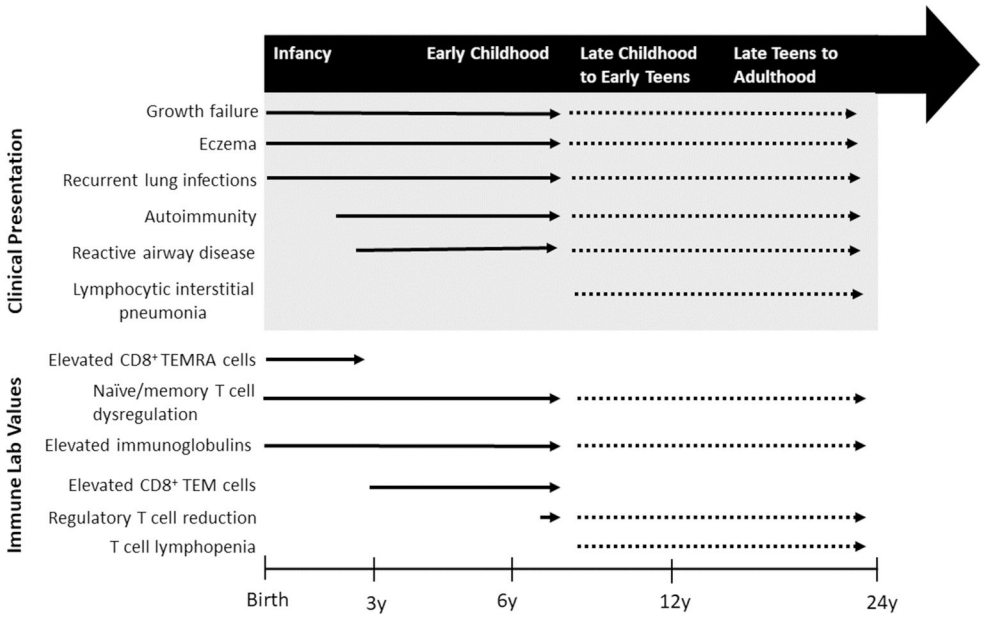

Figure 4: Development of immune dysfunction associated with STAT5B deficiency.

Schematic of clinical presentation and immune values seen in STAT5B deficiency over time. Solid lines indicate characteristics in the three siblings evaluate, and dashed lines indicate previously published data.

Table 1:

Clinical characteristics of siblings compared to typical STAT5B deficient patients

	TYPICAL STAT5B DEFICIENT PATIENT	P3	P2	P1
Endocrine	Severe short stature, facial dysmorphism	Severe short stature, facial dysmorphism	Severe short stature, facial dysmorphism	Severe short stature, facial dysmorphism
Autoimmunity	Variable (alopecia, autoimmune thyroiditis, juvenile idiopathic arthritis, idiopathic thrombocytopenia)	Autoimmune thyroiditis	Autoimmune thyroiditis	Autoimmune thyroiditis, idiopathic thrombocytopenia
Infections	Hemorrhagic varicella, herpes zoster; chronic skin and respiratory infections	Chronic skin and respiratory infections	Chronic skin, urinary tract, and respiratory infections	Chronic urinary tract and respiratory infections
Skin	Eczema	Atopic eczema, skin infections	Atopic eczema, skin infections	Atopic eczema
Pulmonary	Lymphocytic Interstitial Pneumonia	Asthma/Reactive Airway Disease	Asthma/Reactive Airway Disease	Asthma/Reactive Airway Disease; Bronchopulmonary dysplasia
GI	Chronic diarrhea (infancy)	Gastroesophageal reflux disease	Gastroenteritis	Gastroesophageal reflux disease

Table 2:

B cell and Immunoglobulin serum levels in STAT5B deficient siblings

	P3			P2				P1			
Age	**7mo**	**3**	**4**	**2**	**3**	**4**	**5**	**5**	**6**	**7**	**8**
CD19 percentage (range)	23 (15–39)	16 (14–44)	18 (14–44)	33 (14–44)	29 (14–44)	29 (14–44)	32 (10–31)	28 (14–44)	25 (10–31)	26 (10–31)	25 (10–31)
CD19 absolute number (range)	706 (600–2700)	1162 (200–2100)	901 (200–2100)	1318 (200–2100)	615 (200–2100)	1064 (200–2100)	1024 (200–1600)	865 (200–2100)	482 (200–1600)	584 (200–1600)	502 (200–1600)
IgG (mg/dL) (range)	1020 (400–1250)	n/a	1250 (560–1307)	960 (400–1250)	1120 (400–1250)	n/a	1130 (560–1307)	1000 (560–1307)	1210 (560–1307)	n/a	1120 (598–1379)
IgA (mg/dL) (range)	76 (14–105)	n/a	156 (25–152)	55 (14–122)	60.5 (22–157)	n/a	84.1 (25–152)	69.7 (25–152)	77.7 (25–152)	n/a	65 (33–200)
IgM (mg/dL) (range)	100 (41–164)	n/a	102 (41–186)	63 (46–160)	58 (45–190)	n/a	51 (41–186)	83 (41–186)	80 (41–186)	n/a	72 (46–197)
IgE (mg/dL) (range)	1131 **H** (2–97)	n/a	107 (2–307)	266 **H** (2–97)	838 **H** (2–199)	n/a	793 **H** (2–307)	1061 **H** (2–307)	594 **H** (2–307)	n/a	1129 **H** (2–403)
Autoantibodies	Anti-thyroglobulin, anti-thyroid peroxidase			Anti-thyroglobulin, anti-thyroid peroxidase				Anti-thyroglobulin, anti-thyroid peroxidase			

Table 3:

General T cell values and recent thymic emigrant levels in three STAT5B deficient siblings

	P3			P2				P1			
Age	**7mo**	**3**	**4**	**2**	**3**	**4**	**5**	**5**	**6**	**7**	**8**
$CD3^+$ absolute number (range)	2140 (1600–6700)	4936 (900–4500)	3109 (900–4500)	2436 (900–4500)	1284 (900–4500)	2478 (900–4500)	1967 (700–4200)	3109 (900–4500)	1314 (700–4200)	1156 (700–4200)	1377 (700–4200)
$CD3^+$ total percentage (range)	63.8 (57.1–80.7)	66.7 (57.1–80.7)	58.6 **L** (62.2–77.0)	62.5 (57.1–80.7)	65.1 (57.1–80.7)	63.8 (62.2–77)	59.2 **L** (62.2–77)	59.4 **L** (62.2–77)	67.8 (62.2–77)	67.2 (62.2–77)	67 (62.2–77)
$CD4^+$ absolute number (range)	1353 (1000–4600)	3594 (500–2400)	2107 (500–2400)	1222 (500–2400)	861 (500–2400)	1959 (500–2400)	1382 (300–2000)	825 (500–2400)	849 (300–2000)	1024 (300–2000)	879 (300–2000)
$CD4^+$ total percentage (range)	56.9 (55.1–70)	68.1 (55.1–70)	63.8 (39.8–69.4)	45.2 **L** (55.1–70)	66.3 (55.1–70)	73.5 **H** (39.8–69.4)	68.0 (39.8–69.4)	42.1 (39.8–69.4)	61.6 (39.8–69.4)	60.7 (39.8–69.4)	60.9 (39.8–69.4)
$CD8^+$ absolute number (range)	716 (400–2100)	1145 (300–1600)	808 (300–1600)	1005 (300–1600)	335 (300–1600)	403 (300–1600)	494 (300–1800)	822 (300–1600)	367 (300–1800)	408 (300–1800)	396 (300–1800)
$CD8^+$ total percentage (range)	23 (15–39)	16 (14–44)	18 (14–44)	44.4 **H** (27.2–38.4)	26 **L** (27.2–38.4)	20.9 **L** (21.6–44.3)	25.8 (21.6–44.3)	51.5 **H** (21.6–44.3)	29.3 (21.6–44.3)	28.6 (21.6–44.3)	29 (21.6–44.3)
CD4:CD8 ratio (range)	1.9 (1.3–3.9)	3.2 (0.9–3.2)	2.6 (0.9–2.9)	1.2 (0.9–2.9)	2.5 (0.9–2.9)	4.8 **H** (0.9–2.9)	2.9 **H** (0.9–2.6)	1 (0.9–2.9)	2.3 (0.9–2.6)	2.5 (0.9–2.6)	2.2 (0.9–2.6)
$CD4^+CD45^+CD31^+$ %	72 **L** (75.6–97.4)	88.7 (75.6–97.4)	83.6 (69.1–95.6)	76.1 (75.6–97.4)	81.4 (75.6–97.4)	82.7 (69.1–95.6)	79.8 (69.1–95.6)	85.3 (69.1–95.6)	87.7 (69.1–95.6)	86.4 (69.1–95.6)	84.4 (69.1–95.6)
$CD4^+CD45^+CD31^-$ %	27.8 (9.8–30.8)	10.3 (9.8–30.8)	15.3 (4.2–30.7)	23.7 (9.8–30.8)	23.7 (9.8–30.8)	16.7 (4.2–30.7)	19.4 (4.2–30.7)	14.2 (4.2–30.7)	10.5 (4.2–30.7)	10.2 (4.2–30.7)	14.8 (4.2–30.7)