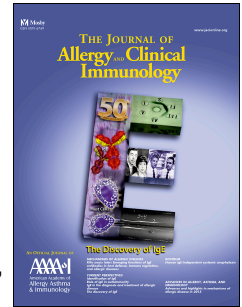


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STAT5B deficiency due to a novel missense mutation in the coiled-coil domain

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We declare no conflicting financial interests.

Capsule summary

A novel homozygous missense mutation in the coiled-coil domain of STAT5B causes an immunodeficiency syndrome characterised by short stature due to GH insensitivity, immune dysregulation and hypothyroidism but currently without pulmonary disease or T-cell lymphopenia.

Key words

STAT5B deficiency, growth failure, GH insensitivity, IGF-I deficiency, immune dysregulation, autoimmunity, eczema, alopecia, hypothyroidism

To the Editor:

We report a 17-year-old boy who presented with growth hormone (GH)-refractory growth failure (Fig.1A), severe eczema (Fig.1B) and autoimmunity. He was born to consanguineous parents at term with normal gestational weight (3.4 kg) and length (51 cm). He initially presented with autoimmune thyroiditis at 4 years of age, with symptoms of lethargy, poor growth, delayed bone age and constipation (Fig.1C), confirmed by high titres of anti-thyroid peroxidase antibodies (>1000 IU/L; normal range: <34 IU/L) and anti-thyroglobulin antibodies (680 IU/L; normal range: <100 IU/L). He was treated with L-thyroxine (levothyroxine), which led to some improvement of his weight and growth. He also suffered from iron-deficiency anaemia, which was treated with Iron (III) hydroxide polymaltose (Maltofer), and ichthyosis from infancy, and developed atopic dermatitis at the age of 7 years. At 9 years of age, he lost his head-hair, eyebrows and eyelashes, and alopecia persisted despite treatment with steroids (Prednisolone) on several occasions; this also failed to resolve his ongoing dermatitis. Three years later he was started on a gluten-free diet after anti-gliadin antibodies were detected in his blood; however, he had no anti-tissue transglutaminase (TTG) antibodies and did not meet the clinical criteria of coeliac disease. Furthermore, as there was no symptomatic improvement the gluten-free diet was stopped after 2 years. Meanwhile, he maintained poor linear growth and delayed puberty. He had normal concentrations of basal GH, but low levels of insulin-like growth factor-1 (IGF-1) (Fig.1D), IgA (Table E1) and vitamin D. In three GH provocation tests he had peak GH levels <10 ng/ml (insulin: 7,35 ng/ml; glucagon: 3,17 ng/ml; arginine: 4,88 ng/ml), which is a response characteristic rather of GH deficiency than of GH insensitivity. However, therapeutic trial with GH for 2 years did not improve growth velocity, and IGF-1 remained below the normal limits throughout the follow-up. Treatment with cyclosporine was started when he was 14 years old and improved the eczema (Fig.1B) and total alopecia, with some relapse of skin rash/thickened dry skin and alopecia after one year. Now he is in a stable condition on cyclosporine therapy, with greatly improved skin status and progress into puberty, although he is still small for his age. Hypothyroidism is well compensated by L-Thyroxine therapy, but he has chronic recurring alopecia.

Assessment of his immunological status revealed a slight reduction in naïve CD4+ T- and B-cells, a striking increase in class-switched memory B cells, abundant activated HLA-DR+ T cells, low levels of IgA and IgM, and increased levels of IgE (Table E1).

In an attempt to identify a presumed autosomal recessive cause of this striking phenotype, we undertook whole exome sequencing and found a novel homozygous missense mutation in the coiled-coil domain (CCD) of the gene *STAT5B* (signal transducer and activator of transcription (STAT) 5B; Fig.2A). The nucleotide change c.452T>C, translating to p.L151P, was confirmed by Sanger sequencing and was found to be heterozygous in both parents and wild-type in the healthy sister (Fig.2B). The mutation is predicted to be deleterious by several prediction programmes (Fig.2C). Indeed, our patient's phenotype was very compatible with that previously described for *STAT5B* deficiency. *STAT5* functions downstream of cytokines such as IL-2, -4, -7, -9, 13, -15, -21, erythropoietin, thrombopoietin and GH by translocating to the nucleus upon receptor engagement and activating transcription of growth factors, transcription factors and other effector molecules. Even though *STAT5A* and *STAT5B* share >90% homology, they have non-redundant as well as redundant functions.¹ The GH receptor preferentially uses *STAT5B* to induce expression of insulin-like growth factor (IGF)-I, which promotes skeletal development and adipogenesis.²

STAT5B also regulates expression of the transcription factor FOXP3 and the high affinity IL-2 receptor subunit CD25, both key molecules in the development and function of regulatory T cells (Tregs).¹ Thus, human STAT5B deficiency is characterised by short stature due to GH insensitivity, and immune dysregulation typically manifest as eczema, chronic diarrhoea, recurrent infections and autoimmune diathesis.² STAT5B-deficient patients have normal levels of GH, but severely reduced serum levels of IGF-I, and are refractory to GH treatment. Other characteristic features are chronic lung diseases such as recurrent pneumonia, lymphocytic interstitial pneumonitis, and/or lung fibrosis, as well as moderate T-cell lymphopenia.^{2,3}

Since the first mutation in STAT5B was reported in 2003, nine more patients with autosomal recessive (AR)² and 13 individuals with autosomal dominant (AD)^{4,5} STAT5B deficiency were identified (Fig.2A). Homozygous mutations were loss-of-function, with five frameshift or nonsense mutations and two missense mutations in the SH2 domain. Both of these missense mutations cause aberrant folding of STAT5B leading to protein aggregation, reduced expression levels and impaired transcriptional activity.^{6,7} AD STAT5B deficiency with variable penetrance was caused by heterozygous missense mutations in the CCD or the DNA-binding domain, with intact expression of a mutant protein with dominant-negative activity (Fig.2A, Table E2).^{4,5} The CCD is important for protein-protein interactions and nuclear import.⁸

To explore the pathogenicity of our patients p.L151P mutation, we first checked for STAT5B protein expression by immunoblotting, showing normal levels in patient EBV-B cells compared to control (Fig.2D). To test if the mutant STAT5B is functionally active, we stimulated PBMCs from the patient and healthy controls with recombinant human IL-2 for 0-90 min and measured STAT5 phosphorylation by flow cytometry. Compared to three controls, the patient showed reduced STAT5 phosphorylation (Fig.2E). As the antibody used recognises phosphorylated STAT5A and STAT5B, it is possible that the remaining signal entirely reflects phosphorylated STAT5A. A similar result was obtained using patient and control EBV-B cells (data not shown). We next investigated Treg numbers and function, which have been shown to be reduced in patients with STAT5B deficiency.² Compared to the travel control, the CD4⁺CD25^{hi}CD127⁻ Treg cell population was reduced by about half in the patient's peripheral blood (Fig.2F). Treg suppressive capacity was assessed with Treg cells from the patient and a travel control (blood shipped for 36h) and an in-house control (fresh blood)(Fig.S1A). Patient Tregs showed significantly reduced suppressive capacity compared to the in-house control ($p=0.0021$), but not the travel control (Fig.S1B). Thus, unfortunately, no conclusion about Treg function can be drawn. T cell proliferation to all mitogens measured was in the normal range (Fig.S1C); albeit perhaps a lower response in the patient was more pronounced for IL-2 (21,894 cpm patient vs 47,107 cpm control) than for other mitogens. The significance of this finding is not clear, but could relate to a defect in STAT5B signalling downstream of the IL-2 receptor.

How a missense mutation in the CCD affects phosphorylation of Y694 in the transcriptional activation domain is interesting to consider. It has been shown for Stat3 that the $\alpha 1$ helix of the CCD, through intramolecular interactions, is crucial for IL-6-induced recruitment of Stat3 to the IL-6 receptor and subsequent Stat3 phosphorylation, nuclear translocation and DNA binding.⁹ Due to high structural conservation between STAT proteins, a similar mechanism might apply to the role of STAT5B's CCD in

recruitment of STAT5B to the IL-2 receptor and subsequent phosphorylation. Interestingly, another STAT5B CCD mutation, Q206R, also inhibits IL-2-induced STAT5B-Y694 phosphorylation.⁴ This heterozygous mutant has a dominant-interfering effect on STAT5B transcriptional function and leads to a phenotype of autoimmunity, lymphoproliferation, granulocytosis and hypogammaglobulinaemia (patient 1), or multiple sclerosis, arthritis and recurrent infections (patient 2), but no GH insensitivity, delayed puberty, eczema or pulmonary disease (Table E2).⁴ In contrast, a further heterozygous STAT5B CCD mutant, Q177P, is robustly phosphorylated, but exerts a dominant-negative effect by abrogating STAT5B nuclear import.⁵ This mutation results in GH-insensitive growth failure, delayed puberty, reduced IGF-1 levels, and severe eczema, as seen in our L151P patient and the four patients with nonsense and frameshift mutations in the CCD.^{2,5} Both our patient and the Q177P index patient had normal instead of elevated levels of prolactin, and raised IgE levels were found in both Q177P and one R152X patients (Table E2).

In summary, this is the first report of a homozygous hypomorphic missense mutation in the STAT5B coiled-coil domain leading to functional defects and a phenotype of STAT5B deficiency. This included GH-refractory growth failure, severe eczema and autoimmune disease but to date without pulmonary problems, severe infections or T-cell lymphopenia. It would have been ideal to treat the GH insensitivity with IGF-1, but unfortunately, that was not available for the patient. We observed a gratifying clinical response of the atopic dermatitis to cyclosporine therapy, coinciding with improved nutritional status, growth and general wellbeing. However the longterm outlook remains guarded. Alternative treatment strategies for immune dysregulation one might consider if symptoms deteriorated include sirolimus therapy or even stem cell transplantation.

For detailed methods, please see the Methods section in this article's Online Repository at www.jacionline.org.

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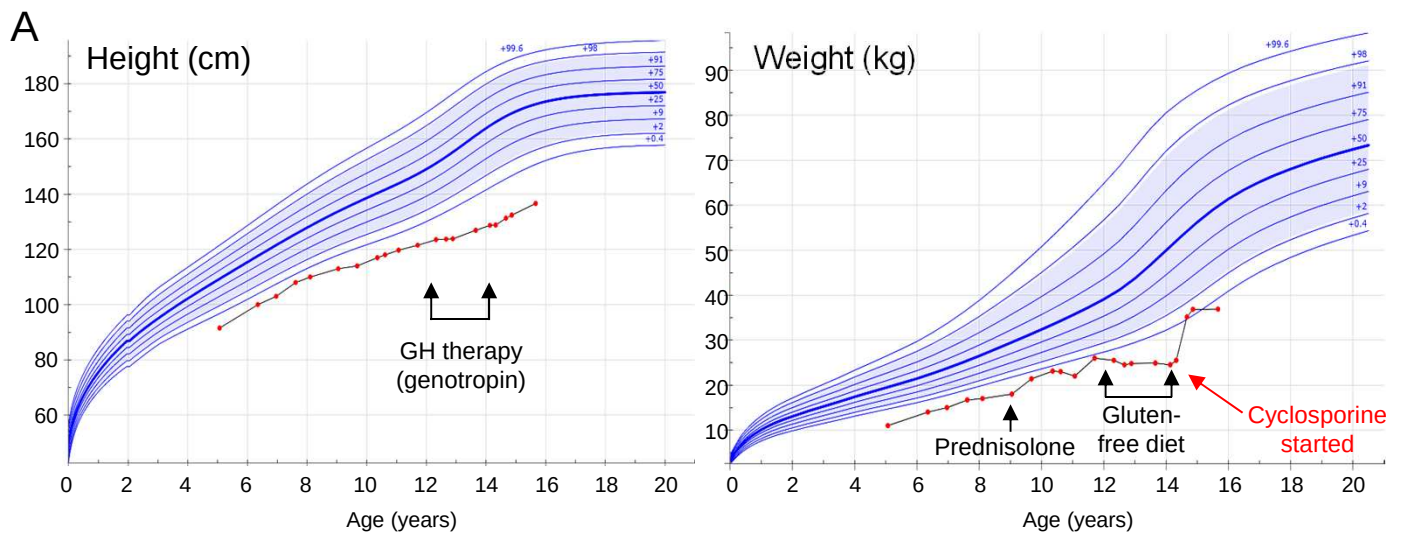
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Figure Legend

Figure 1: Clinical characteristics. A: Height and weight of the patient from age 5-15 years. Periods of growth hormone (GH) therapy, gluten-free diet and steroid treatment are indicated by arrows. B: Eczema and alopecia before (pre) and after (post) cyclosporine treatment. C: Patient clinical features. D: Growth hormone (GH; black line) and Insulin-like Growth Factor (IGF-1; red line) levels in the patient over time. Shaded areas: normal ranges (black: GH; red: IGF-1).

Figure 2: Characterisation of L151P. A: STAT5B schematic showing L151P in the coiled-coil domain. Grey arrows: previously reported pathogenic mutations. Each dot represents one individual with either autosomal recessive (black) or autosomal dominant (blue) mutations. B: Sanger sequencing. C: L151P impact predictions. D: STAT5B immunoblot, EBV-B cells (loading control: GAPDH). E: Phosflow analysis of pSTAT5. PBMCs stimulated with 100ng/ml IL-2 for 0-90 min. Red line: patient; black line: average of 3 healthy controls. Histogram plot: PBMCs gated on live CD3⁺ lymphocytes. Grey area: fluorescence-minus-one (FMO) control; grey line: unstimulated; black line: 15 min stimulation. F: CD4⁺CD25^{hi}CD127⁻ Treg cells in patient and control. Numbers indicate percentages of cells within the gate.



B



C

Age (years)	Clinical Features
Birth	Full term, Dry skin
4	Autoimmune Thyroiditis: - Lethargy - Poor growth - Delayed bone age - Constipation Iron deficiency anaemia Ichthyosis
7	Atopic dermatitis
9	Diffuse alopecia
12	Coeliac-like disease IgA and Vitamin D deficiency
15	GH-refractory growth failure Delayed puberty Severe eczema

D

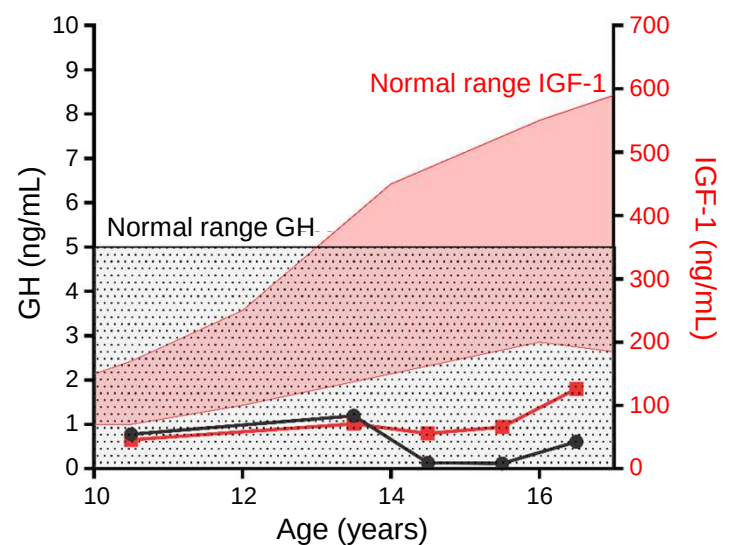


Figure 1

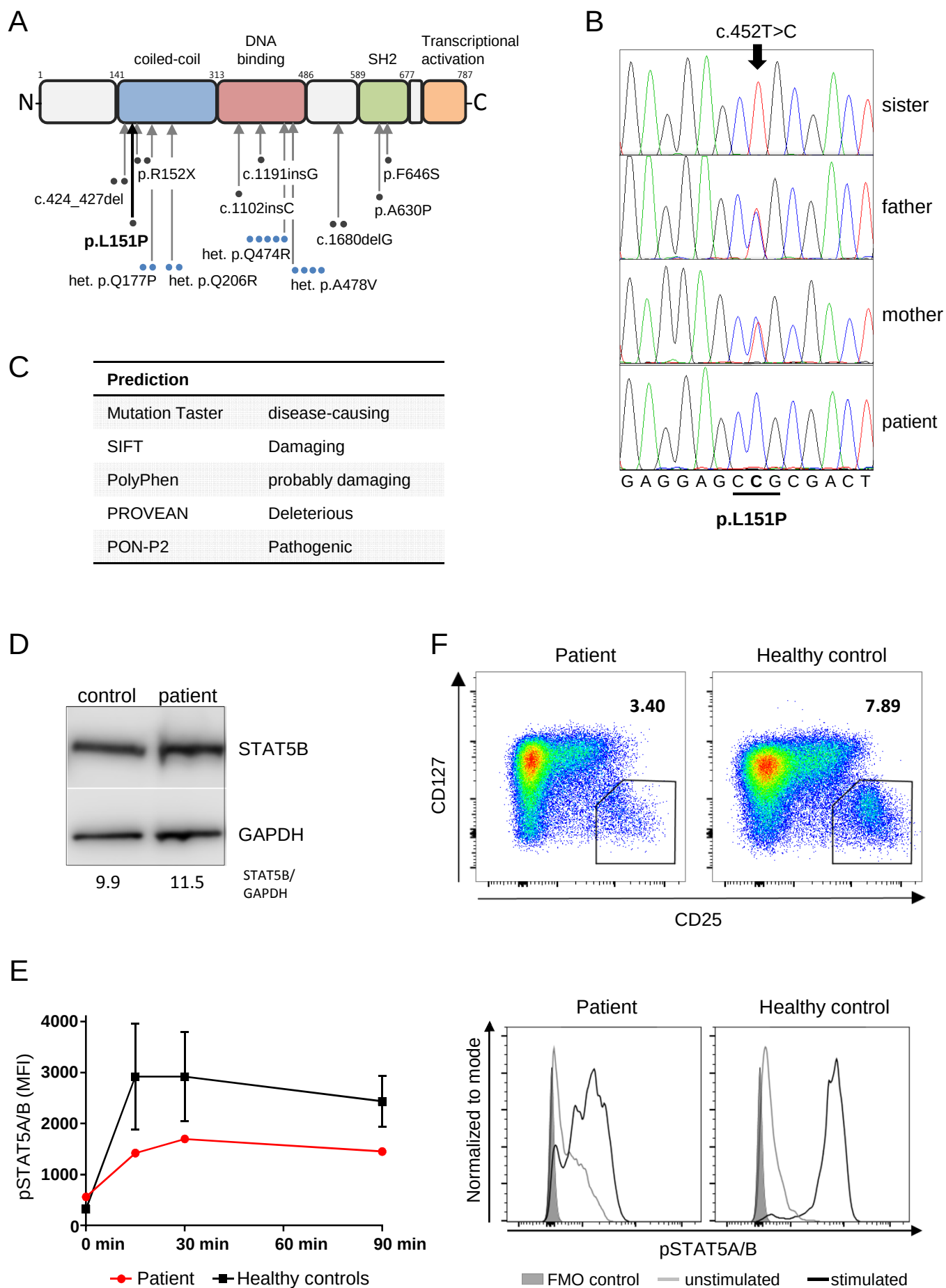


Figure 2

ONLINE REPOSITORY MATERIALTable E1: Laboratory evaluation of STAT5B^{L151P} patient

Parameter	Patient	Reference range ¹ (11-14 years)
Neutrophils	2810 cells/ μ l	1500-8000 cells/ μ l
Lymphocytes	3170 cells/ μ l	1700-6900 cells/ μ l
CD3+	2995 cells/ μ l	900-4500 cells/ μ l
CD4+	2315 cells/ μ l	500-2400 cells/ μ l
CD8+	633 cells/ μ l	300-1600 cells/ μ l
CD19+	270 cells/ μ l	200-2100 cells/ μ l
CD27 ⁺ IgM ⁺ IgD ⁺ (naïve)	51 %	75-87 %
CD27 ⁺ IgM ⁺ IgD ⁺ (memory)	9 %	5-10 %
CD27 ⁺ IgM ⁺ IgD ⁺ (class switch memory)	30 %	3-10 %
CD4 ⁺ /CD45RA ⁺ /CD27 ⁺ (naïve)	240 cells/ μ l (8%)	300-800 cells/ μ l
CD4 ⁺ /CD45RA ⁺ /CD27 ⁺ (naïve)	180 cells/ μ l (6%)	150-400 cells/ μ l
CD4 ⁺ /CD45RA ⁺ /CD27 ⁺ (effector)	60 cells/ μ l (2%)	<60 cells/ μ l
Activated T cells (HLA-DR ⁺)	56 %	<30 %
NK cells (CD16 ⁺ /CD56 ⁺)	205 cells/ μ l (6%)	70-1200 cells/ μ l
IgA	0.1 g/L	0.14-1.23 g/L
IgM	0.43 g/L	0.48-1.68 g/L
IgG	10.1 g/L	4.24-10.51 g/L
IgE	491 IU/mL	0-200 IU/mL
Tetanus antibody	0.31 IU/mL	0.1-10 IU/mL
Haemophilus B antibody	0.2 mg/L	1-20 mg/L
Pneumococcal serotypes: protective responses	8/12 serotypes	
Measles IgG	Detected	
Varicella Zoster IgG	Detected	
Toxoplasma IgG	Detected	
HIV screen	Negative	
Hepatitis screen	Negative	
IGF-1	62.5 ng/mL	143-693 ng/mL
TSH	4.72 μ IU/mL	0.4-4 μ IU/mL
Prolactin	95.4 μ IU/ml	53-360 μ IU/ml

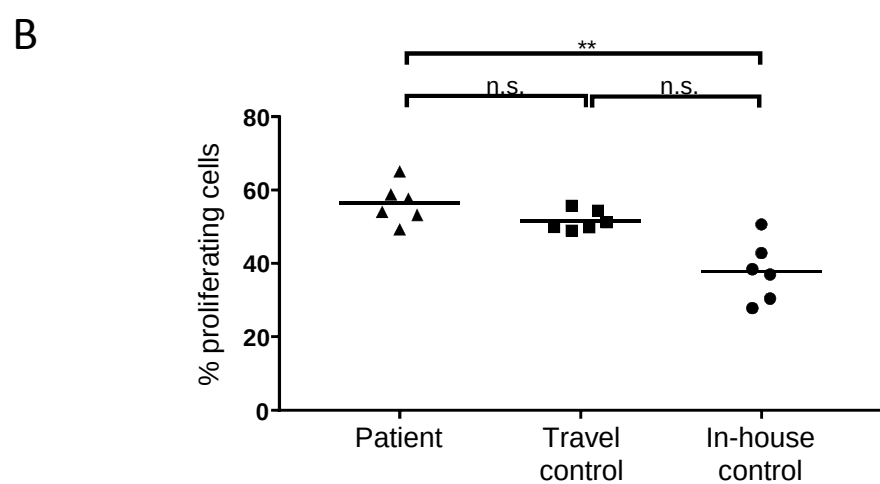
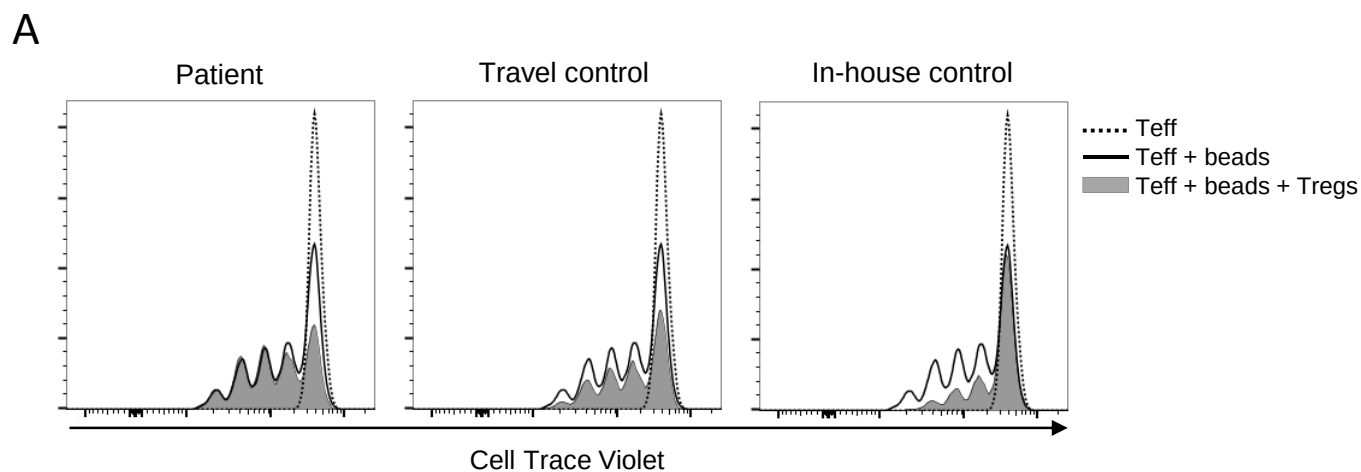
¹ in-house reference range

	L151P (n=1)	Q177P (n=2)	Q206R (n=2)	R152X (n=2)	c.424_427del (n=2)
Mutation	Homozygous Missense	Heterozygous Missense	Heterozygous Missense	Homozygous Nonsense	Homozygous Indel, frameshift (p.L142fsX161)
STAT5B protein expression	Normal	Normal	Normal	Absent	Unknown (expected: absent)

Table E2: Comparative table of coiled-coil domain mutations in STAT5B

STAT5 phosphorylation	Reduced upon IL-2 stimulation of PBMCs	Increased upon GH and IFN- γ stimulation of fibroblasts; defective nuclear import of STAT5B	Reduced upon IL-2, IL-7, or IL-15 stimulation of T-cell blasts	Reduced upon IL-2 stimulation of PBMCs	Unknown
Treg cells	Reduced numbers of CD4 ⁺ CD25 ^{hi} CD127 Tregs	Unknown	Normal frequency of CD4 ⁺ FoxP3 ⁺ Tregs, diminished CD25 expression (1/1)	Reduced numbers of CD4 ⁺ CD25 ^{hi} Tregs (1/1)	Unknown
Postnatal growth failure	Yes	Yes (2/2)	No	Yes (2/2)	Yes (2/2)
Delayed puberty	Yes	Yes (2/2)	No	Yes (2/2)	Yes (1/1)
Basal GH (ng/ml) [<10 ng/ml]	Normal (0.6-1.2)	Normal (1/1) (0.4)	Unknown	Normal (2/2) (6.6; P1) (1.8; P2)	Normal (2/2) (1.7; P1) (1; P2)
Basal IGF-1 (ng/ml) [95-400]	Reduced (46-126)	Reduced (2/2) (56; P1) (58; P2)	Unknown	Not detectable (2/2)	Reduced (2/2) (34; P1) (<25 ; P2)
IgE	Raised	Raised (2/2)	Unknown	Raised (1/1)	Normal (2/2)
Prolactin	Normal	Normal (1/1)	Unknown	Raised (1/1)	Raised (2/2)
Eczema	Severe	Severe (1/1)	No	Severe (2/2)	Present (2/2)
Autoimmunity	Autoimmune thyroiditis, alopecia	No	Immune thrombocytopenic purpura (P1)	No (P1) Autoimmune thyroiditis (P2)	No (P1) Thrombocytopenic purpura (P2)
Chronic pulmonary disease	No	Yes (1/1)	No	Yes (2/2)	Yes (2/2)
Viral infections	No	Unknown	EBV	VZV (2/2)	Unknown
Others	Celiac-like disease Iron-deficiency anaemia Vitamin D deficiency Reduced IgA	Asthma	Splenomegaly, Lymphadenopathy, Necrotizing granulomas/ granulocytosis Hypogammaglobulinaemia (P1) Multiple sclerosis, arthritis, recurrent infections (P2)	Chronic diarrhoea	
Reference		(Klammt et al., 2018)	(Majri et al., 2017)	(Bernasconi et al., 2006; Bezrodnik et al., 2015)	(Pugliese-Pires et al., 2010)

P1: patient 1; P2: patient 2; (1/1): 1 of 1 patient analysed; (2/2): 2 of 2 patients analysed



C

Mitogen	cpm patient	cpm control	S.I. patient	S.I. control
Control	571	313	-	-
PHA	342,510	292,467	x600	x934
ConA	116,591	84,616	x204	x270
PMA	132,537	73,488	x232	x235
PMA + ionophore	175,856	140,270	x308	x448
Anti-CD3	284,488	224,890	x498	x718
Anti-CD3 + IL-2	380,902	303,007	x667	x968
IL-2	21,894	47,107	x38	x151

Figure S1

ONLINE REPOSITORY MATERIAL

METHODS

Study subjects. Patients and their relatives provided written informed consent to participate in research protocols approved by the Newcastle and North Tyneside 1 Research Ethics Committee. Whole blood samples were obtained from these individuals, and genomic DNA was isolated using the DNeasy or QIAamp DNA mini kit (Qiagen).

PCR and sequencing analysis. Specific primers were designed in Primer3web version 4.0.0 (<http://bioinfo.ut.ee/primer3/>). Primer sequences are available on request. Capillary sequencing was performed according to standard methods. Sequences were aligned with the consensus coding sequence (human genome assembly 38) in nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/blast/>). ChromasLite Version 2.1.1 was used for visualization of the sequences.

Whole exome sequencing. Whole exome of the patient was captured using the Agilent SureSelect V5 kit and a library subsequently prepared for paired-end sequencing. This was carried out on an Illumina NextSeq instrument, on a high-output flowcell by OGT (Oxford Gene Technology, UK). Sequencing reads were assessed for quality using the FastQC and MultiQC tools.^{1,2} Reads were aligned to the hg19 assembly of the human reference genome, and prepared for variant calling. Exomes were analysed according to GATK best practices³, as follows: the raw alignment was realigned around indels, quality scores recalibrated (BQSR), and duplicates marked using the Picard suite. Genotyping was performed using a combination of the HaplotypeCaller from GATK in gVCF mode, and JointCalling program leveraging a catalogue of Exome samples from other PID patients. To avoid false positive calls, VQSR was performed on the raw variant callsets for indels and SNPs. Downstream analysis was carried out using the web-based analytics application Ingenuity Variant Analysis (Qiagen).

Cell lines. Epstein Barr Virus (EBV)-immortalized B-cell lines were generated from PBMCs after Ficoll-purification from whole blood (EDTA). Cells were incubated with EBV-containing medium for 1 hour initially, and then in the presence of 1 µg/ml cyclosporine A (CysA) for a further 24h at 37°C, 5% CO₂. Cells were then transferred to fresh 10%FCS/CysA-containing RPMI and incubated until foci of immortalized cells appeared.

Immunoblotting. Cell lysates were prepared from patient and control EBV-B cells. Equal amounts of lysate were separated by electrophoresis on a 4-12% polyacrylamide gradient gel in MOPS buffer (NuPAGE™, Novex) and transferred to a PVDF membrane (Immobilon-P, Millipore). STAT5B was probed using rabbit-anti-human STAT5B monoclonal antibody (EPR16671, Abcam) and anti-rabbit IgG, HRP-

linked secondary antibody (Cell signalling technology, 7074P2). Anti-GAPDH antibody (Cell signalling technology, 5174S) was used as a loading control.

Flow cytometry. Whole blood (EDTA) was rested for 2 hours at room temperature and then stimulated for 0, 15, 30 and 90 minutes at 37°C with 0.1µg/ml recombinant human IL-2 (rhIL-2)(Peprotech, 200-02). Alternatively, EBV-B cells were serum-starved for 1 hour and then stimulated with 0.5µg/ml rhIL-2. Whole blood cells were stained for the surface marker CD3 and intracellularly for phosphorylated STAT5 (pSTAT5), EBV-B cells for pSTAT5 only. Samples were acquired using a FACSCanto II cytometer (BD Biosciences [BD]) and analysed with FlowJo (Treestar). The following antibodies were used: Pacific Blue™ mouse anti-human CD3 (clone UCHT1, BD Biosciences) and Alexa Fluor® 647 mouse anti-human STAT5 (pY694) (clone 47, BD Biosciences).

Lymphocyte proliferation. PBMCs were cultured for 3 days with the respective mitogens and proliferative responses were measured as counts per minute (cpm) of tritiated thymidine incorporated during cell division. Cultures were performed in triplicate with results expressed as the means of the triplicates. The stimulatory index (S.I.) is calculated as stimulated over unstimulated (control) counts.

Regulatory T cell (Treg) suppression assay. CD4+ T cells were enriched using RosetteSep (Stemcell Technologies). To isolate CD4+CD25- effector T cells (Teff), CD25 microbeads and a LD column were used (both Miltenyi Biotec) on a fresh healthy control sample. The Teff cells were labelled with Cell trace violet (Invitrogen) and stimulated with anti-CD2/CD3/CD28-human Treg inspector beads (Miltenyi Biotec) for 4 days. Treg cells from the patient, a travel control (36h old blood samples) and an in-house control (fresh blood sample) were purified via flow sorting (FACS Fusion, BD) gated on CD4+CD25hiCD127- cells. These cells were added at a ratio of 1:1 to the Teff population during the stimulation period. Proliferating Teff cells were evaluated using a FACS Canto flow cytometer (BD). Cultures were performed in sextuplicates.

FIGURE LEGEND

Figure S1: A) Treg suppression assay. Dotted line: effector T cells (Teff) alone. Solid line: Teff stimulated with Treg inspector beads. Shaded area: Teff stimulated with Treg inspector beads in the presence of Tregs. Proliferation of Cell trace violet-labelled Teffs was measured as dilution of the dye with every cell division. Inhibition of Teff proliferation by Tregs is shown by reduced dye dilution. Results are shown for the patient, a travel control (both performed with 36h-old blood) and an in-house control (fresh blood). B) Percentage of proliferating cells with means of six replicates given. Patient Tregs showed significantly reduced suppressive capacity compared to the in-house (**Kruskal-Wallis-Test: $p=0.0021$) but not to the travel control. C) Lymphocyte proliferation, measured as counts per minute (cpm) of incorporated

tritiated thymidine, was normal to all mitogens tested. PHA: phytohaemagglutinin; ConA: concanavalin A; PMA: phorbol myristate acetate; SI: stimulation index.

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