Manuscript Details

Manuscript number	YCLIM_2017_547_R1
Title	A novel de novo activating mutation in STAT3 identified in a patient with common variable immunodeficiency (CVID)
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Abstract

Common variable immunodeficiency (CVID) is characterised by repeated infection associated with primary acquired hypogammaglobulinemia. CVID frequently has a complex aetiology but, in certain cases, it has a monogenic cause. Recently, variants within the gene encoding the transcription factor STAT3 were implicated in monogenic CVID. Here, we describe a patient presenting with symptoms synonymous with CVID, who displayed reduced levels of IgG and IgA, repeated viral infections and multiple additional co-morbidities. Whole-exome sequencing revealed a de novo novel missense mutation in the coiled-coil domain of STAT3 (c.870A>T; p.K290N). Accordingly, the K290N variant of STAT3 was generated, and a STAT3 responsive dual-luciferase reporter assay revealed that the variant strongly enhances STAT3 transcriptional activity both under basal and stimulated (with IL-6) conditions. Overall, these data complement earlier studies in which CVID-associated STAT3 mutations are predicted to enhance transcriptional activity, suggesting that such patients may respond favourably to IL-6 receptor antagonists (e.g. tocilizumab).

Keywords	Common variable immunodeficiency; CVID; hypogammaglobulinemia; STAT3; whole exome sequencing
Taxonomy	Primary Immunodeficiency, Gene Mutation
Manuscript category	Immunogenetics / Genomics

Corresponding Author	Mark Russell
Order of Authors	Mark Russell, Manuela Pigors, maha houssen, Ania Manson, David Kelsell, Hilary Longhurst, Noel Morgan

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

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Dr Mark Russell
University of Exeter Medical School
RILD Building Level 4
Royal Devon & Exeter Hospital (Wonford)
Barrack Road, Exeter EX2 5DW

Phone: +44 (0)1392 408282
Email: m.russell@exeter.ac.uk

Prof George Tsokos
Editor-in-Chief
Clinical Immunology

Dear Professor Tsokos,

Please find enclosed a revised version of manuscript YCLIM_2017_547 entitled 'A novel de novo activating mutation in STAT3 identified in a patient with common variable immunodeficiency (CVID)' submitted to be considered for publication in *Clinical Immunology*. We are delighted with the positive nature of the reviewer's comments, and only very small edits of the submitted documents were required to address these. Please find included point-by-point responses to the reviewer's comments. We have also included a revised version of the manuscript, figures and supplementary tables.

We hope that these responses will be deemed adequate by the editorial and reviewing teams. We look forward to hearing the outcome of your decision.

Yours sincerely,

Mark A. Russell,

Research Fellow

Response to reviewers:

We thank the reviewers of the manuscript for such positive comments, and we are pleased to respond to the minor points which are raised.

Reviewer 1: In Figure 1, the description of the mutation should be A>T rather than A<T.

This has now been amended in the manuscript.

Reviewer 1: I recommend including the vaccination history of the patient.
Reviewer 2: Were vaccine titers checked in this patient? Poor vaccine responses would also help in categorizing this patient's clinical phenotype under CVID.

The patient was started on immunoglobulin replacement for probable CVID prior to referral to specialist immunology services. Test vaccinations were not given. We felt that stopping immunoglobulin replacement to assess vaccination responses could not be justified. The patient received annual inactivated 'flu vaccine. She had received all routine UK childhood vaccinations, namely, diphtheria, tetanus, polio (Sabin), pertussis, measles, mumps, rubella and Bacillus Calmette–Guérin (BCG). A statement in the text (highlighted) has been included to clarify that test vaccinations were not performed, and to ensure that the reader is clear that we have not measured vaccine responses we have included a row in supplementary table 1 stating that this information is not available.

Title; **A novel *de novo* activating mutation in *STAT3* identified in a patient with common variable immunodeficiency (CVID)**

Author list

[a, e]Mark A. Russell, [b,e]Manuela Pigors, [a,c,e] Maha E. Houssen, [d]Ania Manson, [b]David Kelsell, ,[d]Hilary Longhurst, [a]Noel G. Morgan.

Affiliations

a. Institute of Biomedical and Clinical Science, University of Exeter, Exeter, UK.

b. Centre for Cell Biology & Cutaneous Research, Blizard Institute, Queen Mary University of London, UK.

c. Biochemistry Department, Faculty of Pharmacy, Damanhour University, Egypt

d. Barts Health NHS Trust, London, UK

e. These authors contributed equally

Corresponding Author:

Mark A Russell

Institute of Biomedical and Clinical Science,

University of Exeter,

Exeter, EX2 5DW

UK.

Telephone: +44 (0)1392 408282

Email: m.russell@exeter.ac.uk

Abstract

Common variable immunodeficiency (CVID) is characterised by repeated infection associated with primary acquired hypogammaglobulinemia. CVID frequently has a complex aetiology but, in certain cases, it has a monogenic cause. Recently, variants within the gene encoding the transcription factor STAT3 were implicated in monogenic CVID. Here, we describe a patient presenting with symptoms synonymous with CVID, who displayed reduced levels of IgG and IgA, repeated viral infections and multiple additional co-morbidities. Whole-exome sequencing revealed a *de novo* novel missense mutation in the coiled-coil domain of STAT3 (c.870A>T; p.K290N). Accordingly, the K290N variant of *STAT3* was generated, and a STAT3 responsive dual-luciferase reporter assay revealed that the variant strongly enhances STAT3 transcriptional activity both under basal and stimulated (with IL-6) conditions. Overall, these data complement earlier studies in which CVID-associated *STAT3* mutations are predicted to enhance transcriptional activity, suggesting that such patients may respond favourably to IL-6 receptor antagonists (e.g. tocilizumab).

Abbreviations:

CMV, cytomegalovirus; CVID, Common variable immunodeficiency; STAT, signal transducer and activator of transcription; RSV, respiratory syncytial virus.

Keywords:

Common variable immunodeficiency; CVID; hypogammaglobulinemia; STAT3; whole exome sequencing.

1. Introduction

Common variable immunodeficiency (CVID) is the most common severe primary antibody deficiency disorder, characterised by acquired hypogammaglobulinemia and failure to mount an appropriate antibody response to infection or vaccination. CVID patients present with repeated bacterial infections and an increased incidence of certain autoimmune and neoplastic disorders [1]. The disease is thought to have a complex polygenic and multifactorial aetiology in most cases although causative mutations in single genes have been identified in a small proportion of patients [2]. Among the monogenic forms, mutations in *PIK3CD* and *LRBA* comprise approximately half of all reported cases, while mutations in a further 30 additional genes have also been proposed in the remainder [2]. *STAT3* is one such gene and a recent study identified three potential gain-of-function mutations in *STAT3* among patients with CVID [3]. This is consistent with the emerging consensus that the majority of genes associated with monogenic CVID encode signalling proteins (including both receptors and downstream effectors) present in immune cells [2].

STAT3 is a transcription factor whose activity is controlled by numerous cytokines, growth factors and hormones. It becomes phosphorylated on specific tyrosine residues in response to receptor activation and then dimerises before entering the nucleus to regulate the transcription of specific target genes. Interestingly, activating germline mutations in the *STAT3* gene are relatively rare and the majority of such mutations are inactivating and implicated in conditions such as autosomal dominant hyper IgE syndrome [4, 5]. By contrast, where these have been described, the activating variants appear to predispose to various polyautoimmune diseases, including neonatal diabetes mellitus [6-8]. In the current report, we have employed whole exome sequencing of a patient with CVID to reveal a novel, causative, *de novo* mutation in the coiled-coil domain of *STAT3*. Functional studies show that this mutation strongly activates STAT3 under both basal conditions and following stimulation with IL-6, implying that this constitutive activation mediates the disease pathology.

Material and methods

2.1 Whole exome sequencing

After obtaining written informed consent, genomic DNA was extracted from EDTA blood (QiAmp DNA Mini Kit; Qiagen, Hilden, Germany) or saliva samples (prepIT·L2P; DNA Genotek, Ottawa, Canada) from the affected individual and available family members. The project was approved by the East London and City Health Authority Research Ethics Committee and was conducted according to the Declaration of Helsinki Principles.

The blood-derived DNA of the affected individual was subjected to whole exome sequencing. Exome capture and enrichment was performed using the NimbleGen SeqCap EZ Human Exome Library protocol (Roche Nimblegen, Madison, WI, USA). The subsequent DNA library was sequenced with 100 bp paired-end reads on the HiSeq 2000 platform (Illumina, San Diego, CA, USA). Resulting sequence data were aligned to the hg18 human reference genome using the Novoalign alignment tool (Novocraft Technologies Sdn Bhd, Petaling Jaya, Malaysia). Sequence variants were called with SAMtools and annotated via multiple passes through ANNOVAR [9].

2.2 Mutagenesis

The QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) was employed to generate mutations within the human STAT3 gene (Source Bioscience, Nottingham, UK) using manufacturer's guidelines. The primer pairs used to create each mutation were K290N; Fd: TCCCCTTTGTAGGAAACATTTTGCTGCAACTCCTCC Rv: TGGAGGAGTTGCAGCAAAATGTTTCCTACAAAGGGG Y640F; Fd: AGTCCGTGGAACCATTCACAAAGCAGCAGCTG Rv: AGCTGCTGCTTTGTGAATGGTTCCACGGACTG V637M Fd: AGACCCAGATCCAGTCCATGGAACCATACACAAAG, Rv: TGCTTTGTGTATGGTTCCATGGACTGGATCTGGGTC. The STAT3 insert was sequenced to confirm the success of mutagenesis (Source Bioscience). Finally STAT3 inserts were ligated between the AflII and EcoRV restriction sites within the polylinker of a pcDNA5/FRT/TO vector.

2.3 Cell culture

HEK293 cells were cultured in DMEM base media supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37^{o}C and 5% CO_2, and were sub-cultured upon reaching 80% confluence.

2.4 STAT3 Reporter Assay

STAT3 transcriptional activity was investigated using the STAT3 responsive dual firefly/Renilla luciferase Cignal reporter system (Qiagen). Cells were seeded at a density of 1 x 10^5 cells/well in a 24-well plate, and transfected into cells with 200ng of the Cignal reporter construct alongside 400ng of STAT3 constructs using Attractene transfection reagent according to the manufacturer's guidelines (Qiagen). STAT3 activity was assessed 24h after transfection using a dual luciferase reporter system (Promega, Madison, WI, USA).

2.5 Western Blotting

Whole cell protein was extracted from HEK293 cells after lysis [10]. Protein was equalised, then denatured, prior to its loading onto pre-cast 4-12% Bis-Tris polyacrylamide gels (Thermo Fisher Scientific, Boston, MA, USA). Proteins were separated by electrophoresis, before their transfer onto PVDF membrane using a XCell II Blot module (Thermo Fisher Scientific). Immunoblotting was conducted using an iBind Flex Western device according to the manufacturer's instructions (Thermo Fisher Scientific). Membranes were probed initially with antibodies raised against STAT3 (1:1000; Cell Signalling, Beverly, MA, USA; antibody registry: AB_331269) or β-actin (1:2000; Sigma-Aldrich, Poole, UK; antibody registry: AB_476744) and then subsequently with alkaline phosphatase conjugated secondary antibodies raised in the appropriate species. Bands were detected following exposure of membranes to CDP Star chemiluminescent reagent (Sigma-Aldrich) and then visualised using a cDigit blot scanner (LI-COR Biosciences, Lincoln, NE, USA).

Results

3.1 Patient description

The patient was diagnosed with common variable immunodeficiency (CVID) at age 14 on the basis of panhypogammaglobulinaemia and resistant idiopathic thrombocytopaenic purpura, for which splenectomy was performed (Supplementary table 1). She did not receive test vaccination. She had a history of haemolytic anaemia and idiopathic uveitis. Immunoglobulin replacement was started at CVID diagnosis. From early adulthood, the clinical course was complicated by CVID-related inflammatory arthritis, small and large intestinal inflammation, episodes of unexplained ascites, declining respiratory function, chronic hepatic dysfunction and chronic renal impairment. Inflammatory markers were consistently elevated. Furthermore, invasive cytomegalovirus (CMV) with gastric ulcer, typical CMV-related gastric inclusions and viraemia detected on PCR developed while taking mycophenolate and low dose prednisolone, prescribed for the inflammatory complications of CVID. After treatment with intravenous ganciclovir she required ongoing valganciclovir prophylaxis to prevent recurrent CMV viraemia, despite stopping mycophenolate, and maintaining corticosteroid dose at less than 10mg daily.

More recently, at the age of 34 the patient was admitted to hospital with diarrhoea, vomiting, acute on chronic renal failure and was diagnosed with norovirus II. Thereafter the patient had several subsequent hospital admissions with episodes of acute on chronic renal failure, weight loss (8 Kg, 13% of body weight), anorexia, nausea, intermittent diarrhoea and

difficulty maintaining an adequate trough IgG despite high doses of immunoglobulin. Parenteral feeding was commenced to manage the malnutrition that had developed in association with the chronic norovirus infection. During this period persistent rhinovirus was detected in respiratory secretions, and subsequent acute respiratory deterioration was associated with respiratory syncytial virus (RSV) acquisition. The respiratory viruses were successfully treated but despite a high blood level of ribavirin, norovirus was persistently detected in stool. The patient had previously had normal B cell (CD19+) numbers, mildly elevated T cells (CD3+) with inverted CD4+/8+ ratio and raised NK cells (CD16+/56+) in peripheral blood (Supplementary table 2). After contracting norovirus, B cell numbers fell, becoming undetectable in blood and bone marrow after a year. T-cell numbers fell more slowly to below the lower limit of normal and a large granular lymphocyte population (CD57+/CD8+) was evident (Supplementary table 2). The patient eventually succumbed, at the age of 38 years, to a combination of dialysis-dependent renal failure and infection. There was no family history of CVID or autoimmunity, although one sibling had received successful treatment for Hodgkin's lymphoma.

3.2 Identification of mutation

DNA was extracted from the blood or saliva of the patient, her siblings and parents. Variants were identified by whole exome sequencing and filtered for novelty by comparison to dbSNP137, 1000 genomes and an in-house database. From these analyses a heterozygous missense mutation in exon nine of *STAT3* (c.870A>T; [p.K290N] (GenBank: NM_139276)) was identified. The mutation was a *de novo* event and not found in samples taken from either parent or two siblings, as confirmed by Sanger sequencing (Fig 1a). To our knowledge this mutation has not yet been reported in dbSNP, 1000 Genomes, and ExAC Browser. The mutation was signalled as damaging upon analysis by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) with a score of 0.990 and was predicated as disease-causing by Mutation Taster (http://mutationtaster.org/). The K290N mutation resides within the coiled-coil domain of the STAT3 protein, a region which is highly conserved across multiple species (Fig 1b).

3.3 The K290N mutation increases STAT3 activity

To examine the effects of the K290N mutation on STAT3 activity, the mutant form was constructed and transfected into HEK293 cells in parallel with the wildtype. Western blotting analysis revealed a marked increase in STAT3 protein levels relative to cells transfected with empty vector, within 24h. Importantly, transfection with equal quantities of DNA encoding either K290N or wildtype *STAT3* cDNA resulted in similar expression of STAT3 protein (Fig 2a).

A STAT3-specific reporter assay was used to examine the transcriptional activity of the variant and revealed that introduction of the K290N substitution caused a ~16-fold increase in basal activity relative to the wildtype (Fig 2b). Treatment of cells with IL-6 for 24h promoted an increase in wildtype STAT3 activity (by ~19-fold) and led to a further enhancement of STAT3-K290N activity (totalling ~60-fold) above unstimulated wildtype cells, respectively (Fig 2c). Importantly, under these conditions, IL-6 also elicited a marked increase in the activity of the STAT3-K290N mutant beyond that observed in unstimulated cells expressing the mutant form.

In these experiments, previously reported mutations which either enhance (Y640F) or inhibit (V637M) the activity of STAT3 were tested in parallel, to validate the assay system. These forms of STAT3 altered transcriptional activity in the expected directions under both IL-6 stimulated and unstimulated conditions (Fig 2b & c).

Discussion

We demonstrate that a novel *de novo* mutation in *STAT3*, discovered in a patient with CVID, results in a profound increase in transcriptional activity resulting from an amino acid substitution (asparagine for lysine) located at position 290 within the coiled-coil domain of the protein. As such, the present work supports earlier evidence in which activating mutations in *STAT3* were also found in three other patients with CVID [3]. These vary in that they occur at different residues within the protein, but it seems significant that three of the variants (including that identified here) lie within the coiled-coil domain. Furthermore, each promotes the acquisition of gain-of-function properties by enhancing the transcriptional activity of STAT3 [3]. Taken together, the data suggest very strongly that mutations with the coiled-coil domain of STAT3 can promote the transcriptional activity of STAT3 and that this altered phenotype is pathogenic for CVID.

The clinical phenotype of the patient was typical for CVID. The patient presented with reduced IgG and IgA levels, however IgM was within the normal range, these observations are similar to those described in CVID patients with other mutations in STAT3 [3]. In addition, in the current study changes in the immune cell profile were noted, including an elevation in CD3+ T-cells and a reduction in the CD4+/CD8+ cell ratio. Similar profiles have been observed in previously published CVID cases [11]. The patient experienced repeated infections as well as liver and gastrointestinal disease, however malignancy, although commonly reported in CVID patients [1], did not develop. These data highlight the variability of CVID even when caused by mutations within the same gene. In the case of *STAT3*, the

mode of activation probably differs in subtle ways for each mutation leading to the variable patient phenotypes. Such considerations should be included in the planning of the clinical management for a patient with the disease.

The coiled-coil domain plays an important role in regulating the nuclear import of STAT3, and it is known that modification of residues within this domain can disrupt or abrogate nuclear translocation. This process is thought to be regulated by at least two distinct regions within the coiled-coil domain and, in particular, residues located between amino acids 150-163 [12] and at 214-215 have been implicated [13]. Clearly, the mutation we have identified (K290N) lies outside these regions and might not, then, be immediately implicated in altered nuclear localisation. However, changes in the folding of STAT3 arising from the loss of a positively charged lysine residue might influence its nuclear retention and, it is also possible that the mutation leads to the generation of a novel nuclear localisation sequence *per se*. In this context, the use of predictive algorithms (NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) reveals that the K290N mutation generates a novel motif (QNVSYK) at which N-glycosylation is predicted to occur (on the substituted asparagine residue). Such a change in glycosylation status could influence the subcellular localisation of the protein, including facilitation of nuclear entry [14]. In the present work, we have not proven directly that the K290N mutant is preferentially retained in the nucleus although this would be consistent with the activating phenotype and with a recent report documenting sustained nuclear retention as a mechanism for STAT3 activation associated with another activating variant [15].

In support of the present data, others have reported that increased tyrosine phosphorylation of STAT3 (and, therefore, enhanced nuclear import and activation of transcriptional activity) is detectable in the memory B-cell population of patients with CVID [16]. This was associated with elevated apoptotic rates in these cells, both under control conditions and upon stimulation with relevant cytokines, and could provide one mechanism by which B-cells are depleted in the disease. Given that we find a greatly enhanced response to IL-6 in the K290N mutant, IL-6 receptor antagonists, such as tocilizumab, may provide a relatively non-toxic therapeutic option for managing the autoimmunity. Indeed, this approach has been used successfully in other disease contexts, such as rheumatoid arthritis, where activation of STAT3 has been implicated as causative [17, 18].

Finally, it is important to emphasise that, while constitutive activation of STAT3 can lead to the development of CVID, it is also clear that alternative pathological phenotypes can develop from such mutations. For example, certain activating germline mutations in *STAT3*

have been implicated in the early onset of poly-autoimmune phenotypes rather than hypogammaglobulinaemia [6]. Furthermore, somatic mutations leading to STAT3 activation have also been described in patients with rare forms of cancer [19, 20]. It remains to be established why different gain-of-function mutations in *STAT3* cause variable disease phenotypes but this may be related to differences in the degree of activation associated with each mutation and their propensity to alter protein-protein interactions within the signalling cascade.

Acknowledgements

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Figure legends

Figure 1. A *de novo* mutation identified in the coiled-coil domain of STAT3 in a CVID patient. (a) Whole exome sequencing identified a mutation (K290N) within *STAT3* from a patient with CVID, this was absent from parents and siblings of the affected individual. (b) The mutation was located within the coiled-coil domain of STAT3, and was in a highly conserved region of the protein. Other reported STAT3 mutations associated with CVID are also indicated.

Figure 2. Transfection of K290N-STAT3 into cells increased STAT3 activity. (a) HEK293 cells were transfected with equal amounts of an empty vector, wildtype STAT3 or K290N and expression of STAT3 was studied by Western blotting. Results are representative of two separate experiments. (b) Cells were alternatively transfected with wildtype, K290N and mutations which are known to activate (Y640F) and inactivate (V637M) STAT3. These cells were either grown in the presence or absence of 20ng/ml IL-6, and transcriptional activity was determined using a dual-luciferase reporter assay (n = 3-6). ***$p<0.001$.

Highlights:

- A case study of a female patient with common variable immunodeficiency
- A novel *de novo* missense mutation was identified within the coiled-coil region of the *STAT3* gene
- The variant enhanced STAT3 transcriptional activity under basal and stimulated conditions

Title; **A novel *de novo* activating mutation in *STAT3* identified in a patient with common variable immunodeficiency (CVID)**

Author list

[a, e]Mark A. Russell, [b,e]Manuela Pigors, [a,c,e] Maha E. Houssen, [d]Ania Manson, [b]David Kelsell, ,[d]Hilary Longhurst, [a]Noel G. Morgan.

Affiliations

a. Institute of Biomedical and Clinical Science, University of Exeter, Exeter, UK.

b. Centre for Cell Biology & Cutaneous Research, Blizard Institute, Queen Mary University of London, UK.

c. Biochemistry Department, Faculty of Pharmacy, Damanhour University, Egypt

d. Barts Health NHS Trust, London, UK

e. These authors contributed equally

Corresponding Author:

Mark A Russell

Institute of Biomedical and Clinical Science,

University of Exeter,

Exeter, EX2 5DW

UK.

Telephone: +44 (0)1392 408282

Email: m.russell@exeter.ac.uk

Abstract

Common variable immunodeficiency (CVID) is characterised by repeated infection associated with primary acquired hypogammaglobulinemia. CVID frequently has a complex aetiology but, in certain cases, it has a monogenic cause. Recently, variants within the gene encoding the transcription factor STAT3 were implicated in monogenic CVID. Here, we describe a patient presenting with symptoms synonymous with CVID, who displayed reduced levels of IgG and IgA, repeated viral infections and multiple additional co-morbidities. Whole-exome sequencing revealed a *de novo* novel missense mutation in the coiled-coil domain of STAT3 (c.870A>T; p.K290N). Accordingly, the K290N variant of *STAT3* was generated, and a STAT3 responsive dual-luciferase reporter assay revealed that the variant strongly enhances STAT3 transcriptional activity both under basal and stimulated (with IL-6) conditions. Overall, these data complement earlier studies in which CVID-associated *STAT3* mutations are predicted to enhance transcriptional activity, suggesting that such patients may respond favourably to IL-6 receptor antagonists (e.g. tocilizumab).

Abbreviations:

CMV, cytomegalovirus; CVID, Common variable immunodeficiency; STAT, signal transducer and activator of transcription; RSV, respiratory syncytial virus.

Keywords:

Common variable immunodeficiency; CVID; hypogammaglobulinemia; STAT3; whole exome sequencing.

1. Introduction

Common variable immunodeficiency (CVID) is the most common severe primary antibody deficiency disorder, characterised by acquired hypogammaglobulinemia and failure to mount an appropriate antibody response to infection or vaccination. CVID patients present with repeated bacterial infections and an increased incidence of certain autoimmune and neoplastic disorders [1]. The disease is thought to have a complex polygenic and multifactorial aetiology in most cases although causative mutations in single genes have been identified in a small proportion of patients [2]. Among the monogenic forms, mutations in *PIK3CD* and *LRBA* comprise approximately half of all reported cases, while mutations in a further 30 additional genes have also been proposed in the remainder [2]. *STAT3* is one such gene and a recent study identified three potential gain-of-function mutations in *STAT3* among patients with CVID [3]. This is consistent with the emerging consensus that the majority of genes associated with monogenic CVID encode signalling proteins (including both receptors and downstream effectors) present in immune cells [2].

STAT3 is a transcription factor whose activity is controlled by numerous cytokines, growth factors and hormones. It becomes phosphorylated on specific tyrosine residues in response to receptor activation and then dimerises before entering the nucleus to regulate the transcription of specific target genes. Interestingly, activating germline mutations in the *STAT3* gene are relatively rare and the majority of such mutations are inactivating and implicated in conditions such as autosomal dominant hyper IgE syndrome [4, 5]. By contrast, where these have been described, the activating variants appear to predispose to various polyautoimmune diseases, including neonatal diabetes mellitus [6-8]. In the current report, we have employed whole exome sequencing of a patient with CVID to reveal a novel, causative, *de novo* mutation in the coiled-coil domain of *STAT3*. Functional studies show that this mutation strongly activates STAT3 under both basal conditions and following stimulation with IL-6, implying that this constitutive activation mediates the disease pathology.

Material and methods

2.1 Whole exome sequencing

After obtaining written informed consent, genomic DNA was extracted from EDTA blood (QiAmp DNA Mini Kit; Qiagen, Hilden, Germany) or saliva samples (prepIT·L2P; DNA Genotek, Ottawa, Canada) from the affected individual and available family members. The project was approved by the East London and City Health Authority Research Ethics Committee and was conducted according to the Declaration of Helsinki Principles.

The blood-derived DNA of the affected individual was subjected to whole exome sequencing. Exome capture and enrichment was performed using the NimbleGen SeqCap EZ Human Exome Library protocol (Roche Nimblegen, Madison, WI, USA). The subsequent DNA library was sequenced with 100 bp paired-end reads on the HiSeq 2000 platform (Illumina, San Diego, CA, USA). Resulting sequence data were aligned to the hg18 human reference genome using the Novoalign alignment tool (Novocraft Technologies Sdn Bhd, Petaling Jaya, Malaysia). Sequence variants were called with SAMtools and annotated via multiple passes through ANNOVAR [9].

2.2 Mutagenesis

The QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) was employed to generate mutations within the human STAT3 gene (Source Bioscience, Nottingham, UK) using manufacturer's guidelines. The primer pairs used to create each mutation were K290N; Fd: TCCCCTTTGTAGGAAACATTTTGCTGCAACTCCTCC Rv: TGGAGGAGTTGCAGCAAAATGTTTCCTACAAAGGGG Y640F; Fd: AGTCCGTGGAACCATTCACAAAGCAGCAGCTG Rv: AGCTGCTGCTTTGTGAATGGTTCCACGGACTG V637M Fd: AGACCCAGATCCAGTCCATGGAACCATACACAAAG, Rv: TGCTTTGTGTATGGTTCCATGGACTGGATCTGGGTC. The STAT3 insert was sequenced to confirm the success of mutagenesis (Source Bioscience). Finally STAT3 inserts were ligated between the AflII and EcoRV restriction sites within the polylinker of a pcDNA5/FRT/TO vector.

2.3 Cell culture

HEK293 cells were cultured in DMEM base media supplemented with 10% foetal bovine serum, 2mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37^{o}C and 5% CO_2, and were sub-cultured upon reaching 80% confluence.

2.4 STAT3 Reporter Assay

STAT3 transcriptional activity was investigated using the STAT3 responsive dual firefly/Renilla luciferase Cignal reporter system (Qiagen). Cells were seeded at a density of 1 x 10^5 cells/well in a 24-well plate, and transfected into cells with 200ng of the Cignal reporter construct alongside 400ng of STAT3 constructs using Attractene transfection reagent according to the manufacturer's guidelines (Qiagen). STAT3 activity was assessed 24h after transfection using a dual luciferase reporter system (Promega, Madison, WI, USA).

2.5 Western Blotting

Whole cell protein was extracted from HEK293 cells after lysis [10]. Protein was equalised, then denatured, prior to its loading onto pre-cast 4-12% Bis-Tris polyacrylamide gels (Thermo Fisher Scientific, Boston, MA, USA). Proteins were separated by electrophoresis, before their transfer onto PVDF membrane using a XCell II Blot module (Thermo Fisher Scientific). Immunoblotting was conducted using an iBind Flex Western device according to the manufacturer's instructions (Thermo Fisher Scientific). Membranes were probed initially with antibodies raised against STAT3 (1:1000; Cell Signalling, Beverly, MA, USA; antibody registry: AB_331269) or β-actin (1:2000; Sigma-Aldrich, Poole, UK; antibody registry: AB_476744) and then subsequently with alkaline phosphatase conjugated secondary antibodies raised in the appropriate species. Bands were detected following exposure of membranes to CDP Star chemiluminescent reagent (Sigma-Aldrich) and then visualised using a cDigit blot scanner (LI-COR Biosciences, Lincoln, NE, USA).

Results

3.1 Patient description

The patient was diagnosed with common variable immunodeficiency (CVID) at age 14 on the basis of panhypogammaglobulinaemia and resistant idiopathic thrombocytopaenic purpura, for which splenectomy was performed (Supplementary table 1). She had a history of haemolytic anaemia and idiopathic uveitis. Immunoglobulin replacement was started at CVID diagnosis. From early adulthood, the clinical course was complicated by CVID-related inflammatory arthritis, small and large intestinal inflammation, episodes of unexplained ascites, declining respiratory function, chronic hepatic dysfunction and chronic renal impairment. Inflammatory markers were consistently elevated. Furthermore, invasive cytomegalovirus (CMV) with gastric ulcer, typical CMV-related gastric inclusions and viraemia detected on PCR developed while taking mycophenolate and low dose prednisolone, prescribed for the inflammatory complications of CVID. After treatment with intravenous ganciclovir she required ongoing valganciclovir prophylaxis to prevent recurrent CMV viraemia, despite stopping mycophenolate, and maintaining corticosteroid dose at less than 10mg daily.

More recently, at the age of 34 the patient was admitted to hospital with diarrhoea, vomiting, acute on chronic renal failure and was diagnosed with norovirus II. Thereafter the patient had several subsequent hospital admissions with episodes of acute on chronic renal failure, weight loss (8 Kg, 13% of body weight), anorexia, nausea, intermittent diarrhoea and

difficulty maintaining an adequate trough IgG despite high doses of immunoglobulin. Parenteral feeding was commenced to manage the malnutrition that had developed in association with the chronic norovirus infection. During this period persistent rhinovirus was detected in respiratory secretions, and subsequent acute respiratory deterioration was associated with respiratory syncytial virus (RSV) acquisition. The respiratory viruses were successfully treated but despite a high blood level of ribavirin, norovirus was persistently detected in stool. The patient had previously had normal B cell (CD19+) numbers, mildly elevated T cells (CD3+) with inverted CD4+/8+ ratio and raised NK cells (CD16+/56+) in peripheral blood (Supplementary table 2). After contracting norovirus, B cell numbers fell, becoming undetectable in blood and bone marrow after a year. T-cell numbers fell more slowly to below the lower limit of normal and a large granular lymphocyte population (CD57+/CD8+) was evident (Supplementary table 2). The patient eventually succumbed, at the age of 38 years, to a combination of dialysis-dependent renal failure and infection. There was no family history of CVID or autoimmunity, although one sibling had received successful treatment for Hodgkin's lymphoma.

3.2 Identification of mutation

DNA was extracted from the blood or saliva of the patient, her siblings and parents. Variants were identified by whole exome sequencing and filtered for novelty by comparison to dbSNP137, 1000 genomes and an in-house database. From these analyses a heterozygous missense mutation in exon nine of *STAT3* (c.870A>T; [p.K290N] (GenBank: NM_139276)) was identified. The mutation was a *de novo* event and not found in samples taken from either parent or two siblings, as confirmed by Sanger sequencing (Fig 1a). To our knowledge this mutation has not yet been reported in dbSNP, 1000 Genomes, and ExAC Browser. The mutation was signalled as damaging upon analysis by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) with a score of 0.990 and was predicated as disease-causing by Mutation Taster (http://mutationtaster.org/). The K290N mutation resides within the coiled-coil domain of the STAT3 protein, a region which is highly conserved across multiple species (Fig 1b).

3.3 The K290N mutation increases STAT3 activity

To examine the effects of the K290N mutation on STAT3 activity, the mutant form was constructed and transfected into HEK293 cells in parallel with the wildtype. Western blotting analysis revealed a marked increase in STAT3 protein levels relative to cells transfected with empty vector, within 24h. Importantly, transfection with equal quantities of DNA encoding either K290N or wildtype *STAT3* cDNA resulted in similar expression of STAT3 protein (Fig 2a).

A STAT3-specific reporter assay was used to examine the transcriptional activity of the variant and revealed that introduction of the K290N substitution caused a ~16-fold increase in basal activity relative to the wildtype (Fig 2b). Treatment of cells with IL-6 for 24h promoted an increase in wildtype STAT3 activity (by ~19-fold) and led to a further enhancement of STAT3-K290N activity (totalling ~60-fold) above unstimulated wildtype cells, respectively (Fig 2c). Importantly, under these conditions, IL-6 also elicited a marked increase in the activity of the STAT3-K290N mutant beyond that observed in unstimulated cells expressing the mutant form.

In these experiments, previously reported mutations which either enhance (Y640F) or inhibit (V637M) the activity of STAT3 were tested in parallel, to validate the assay system. These forms of STAT3 altered transcriptional activity in the expected directions under both IL-6 stimulated and unstimulated conditions (Fig 2b & c).

Discussion

We demonstrate that a novel *de novo* mutation in *STAT3*, discovered in a patient with CVID, results in a profound increase in transcriptional activity resulting from an amino acid substitution (asparagine for lysine) located at position 290 within the coiled-coil domain of the protein. As such, the present work supports earlier evidence in which activating mutations in *STAT3* were also found in three other patients with CVID [3]. These vary in that they occur at different residues within the protein, but it seems significant that three of the variants (including that identified here) lie within the coiled-coil domain. Furthermore, each promotes the acquisition of gain-of-function properties by enhancing the transcriptional activity of STAT3 [3]. Taken together, the data suggest very strongly that mutations with the coiled-coil domain of STAT3 can promote the transcriptional activity of STAT3 and that this altered phenotype is pathogenic for CVID.

The clinical phenotype of the patient was typical for CVID. The patient presented with reduced IgG and IgA levels, however IgM was within the normal range, these observations are similar to those described in CVID patients with other mutations in STAT3 [3]. In addition, in the current study changes in the immune cell profile were noted, including an elevation in CD3+ T-cells and a reduction in the CD4+/CD8+ cell ratio. Similar profiles have been observed in previously published CVID cases [11]. The patient experienced repeated infections as well as liver and gastrointestinal disease, however lung disease and malignancy, although commonly reported in CVID patients [1], did not develop. These data highlight the variability of CVID even when caused by mutations within the same gene. In the

case of *STAT3*, the mode of activation probably differs in subtle ways for each mutation leading to the variable patient phenotypes. Such considerations should be included in the planning of the clinical management for a patient with the disease.

The coiled-coil domain plays an important role in regulating the nuclear import of STAT3, and it is known that modification of residues within this domain can disrupt or abrogate nuclear translocation. This process is thought to be regulated by at least two distinct regions within the coiled-coil domain and, in particular, residues located between amino acids 150-163 [12] and at 214-215 have been implicated [13]. Clearly, the mutation we have identified (K290N) lies outside these regions and might not, then, be immediately implicated in altered nuclear localisation. However, changes in the folding of STAT3 arising from the loss of a positively charged lysine residue might influence its nuclear retention and, it is also possible that the mutation leads to the generation of a novel nuclear localisation sequence *per se*. In this context, the use of predictive algorithms (NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) reveals that the K290N mutation generates a novel motif (QNVSYK) at which N-glycosylation is predicted to occur (on the substituted asparagine residue). Such a change in glycosylation status could influence the subcellular localisation of the protein, including facilitation of nuclear entry [14]. In the present work, we have not proven directly that the K290N mutant is preferentially retained in the nucleus although this would be consistent with the activating phenotype and with a recent report documenting sustained nuclear retention as a mechanism for STAT3 activation associated with another activating variant [15].

In support of the present data, others have reported that increased tyrosine phosphorylation of STAT3 (and, therefore, enhanced nuclear import and activation of transcriptional activity) is detectable in the memory B-cell population of patients with CVID [16]. This was associated with elevated apoptotic rates in these cells, both under control conditions and upon stimulation with relevant cytokines, and could provide one mechanism by which B-cells are depleted in the disease. Given that we find a greatly enhanced response to IL-6 in the K290N mutant, IL-6 receptor antagonists, such as tocilizumab, may provide a relatively non-toxic therapeutic option for managing the autoimmunity. Indeed, this approach has been used successfully in other disease contexts, such as rheumatoid arthritis, where activation of STAT3 has been implicated as causative [17, 18].

Finally, it is important to emphasise that, while constitutive activation of STAT3 can lead to the development of CVID, it is also clear that alternative pathological phenotypes can develop from such mutations. For example, certain activating germline mutations in *STAT3*

have been implicated in the early onset of poly-autoimmune phenotypes rather than hypogammaglobulinaemia [6]. Furthermore, somatic mutations leading to STAT3 activation have also been described in patients with rare forms of cancer [19, 20]. It remains to be established why different gain-of-function mutations in *STAT3* cause variable disease phenotypes but this may be related to differences in the degree of activation associated with each mutation and their propensity to alter protein-protein interactions within the signalling cascade.

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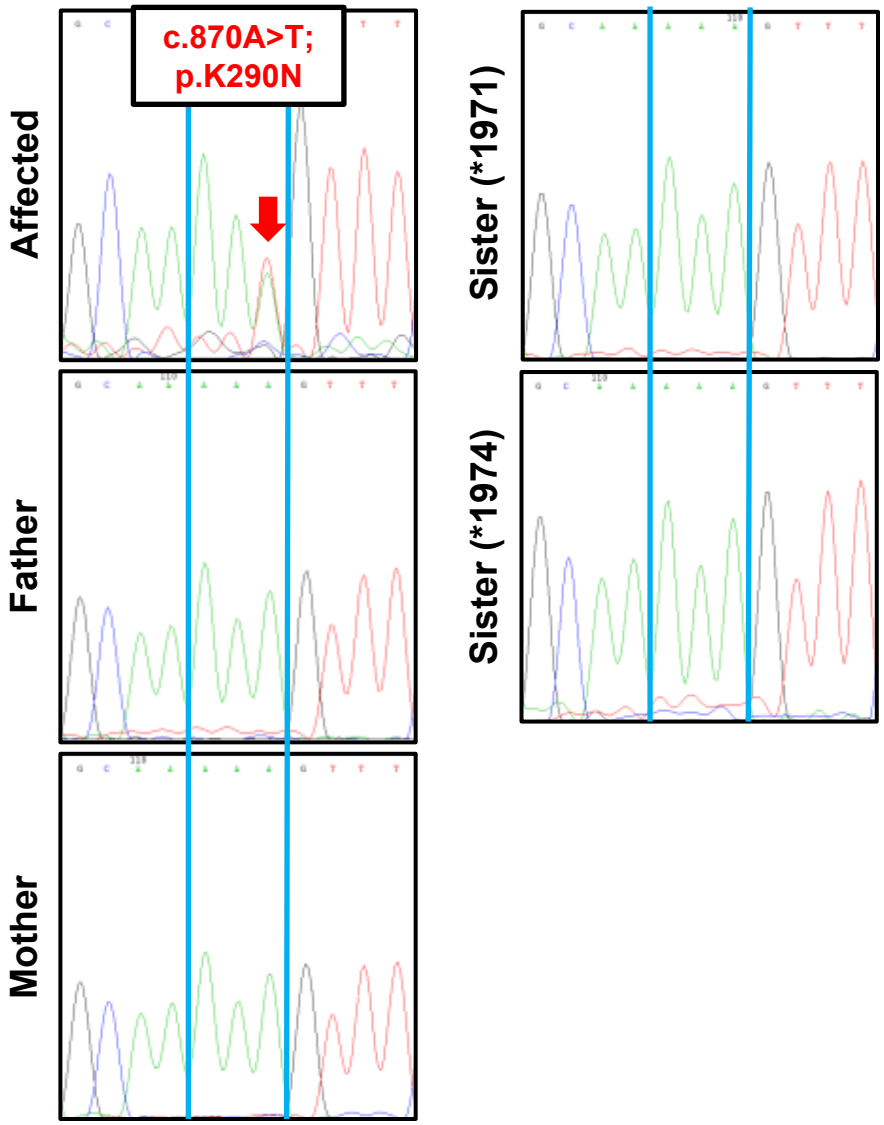
Figure legends

Figure 1. A *de novo* mutation identified in the coiled-coil domain of STAT3 in a CVID patient. (a) Whole exome sequencing identified a mutation (K290N) within *STAT3* from a patient with CVID, this was absent from parents and siblings of the affected individual. (b) The mutation was located within the coiled-coil domain of STAT3, and was in a highly conserved region of the protein. Other reported STAT3 mutations associated with CVID are also indicated.

Figure 2. Transfection of K290N-STAT3 into cells increased STAT3 activity. (a) HEK293 cells were transfected with equal amounts of an empty vector, wildtype STAT3 or K290N and expression of STAT3 was studied by Western blotting. Results are representative of two separate experiments. (b) Cells were alternatively transfected with wildtype, K290N and mutations which are known to activate (Y640F) and inactivate (V637M) STAT3. These cells were either grown in the presence or absence of 20ng/ml IL-6, and transcriptional activity was determined using a dual-luciferase reporter assay (n = 3-6). ***$p<0.001$.

Fig 1

(a)


c.870A>T;
p.K290N
Affected
Father
Mother
Sister (*1971)
Sister (*1974)

(b)

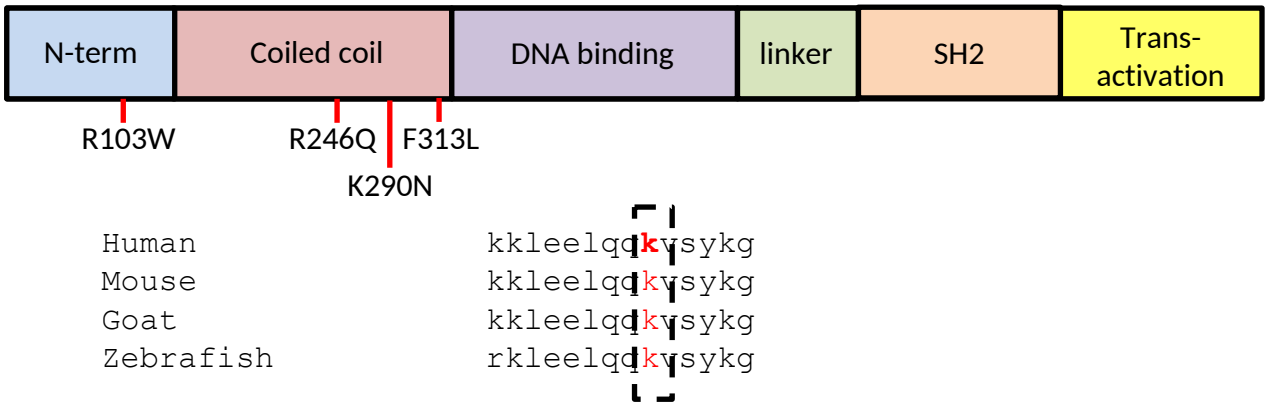
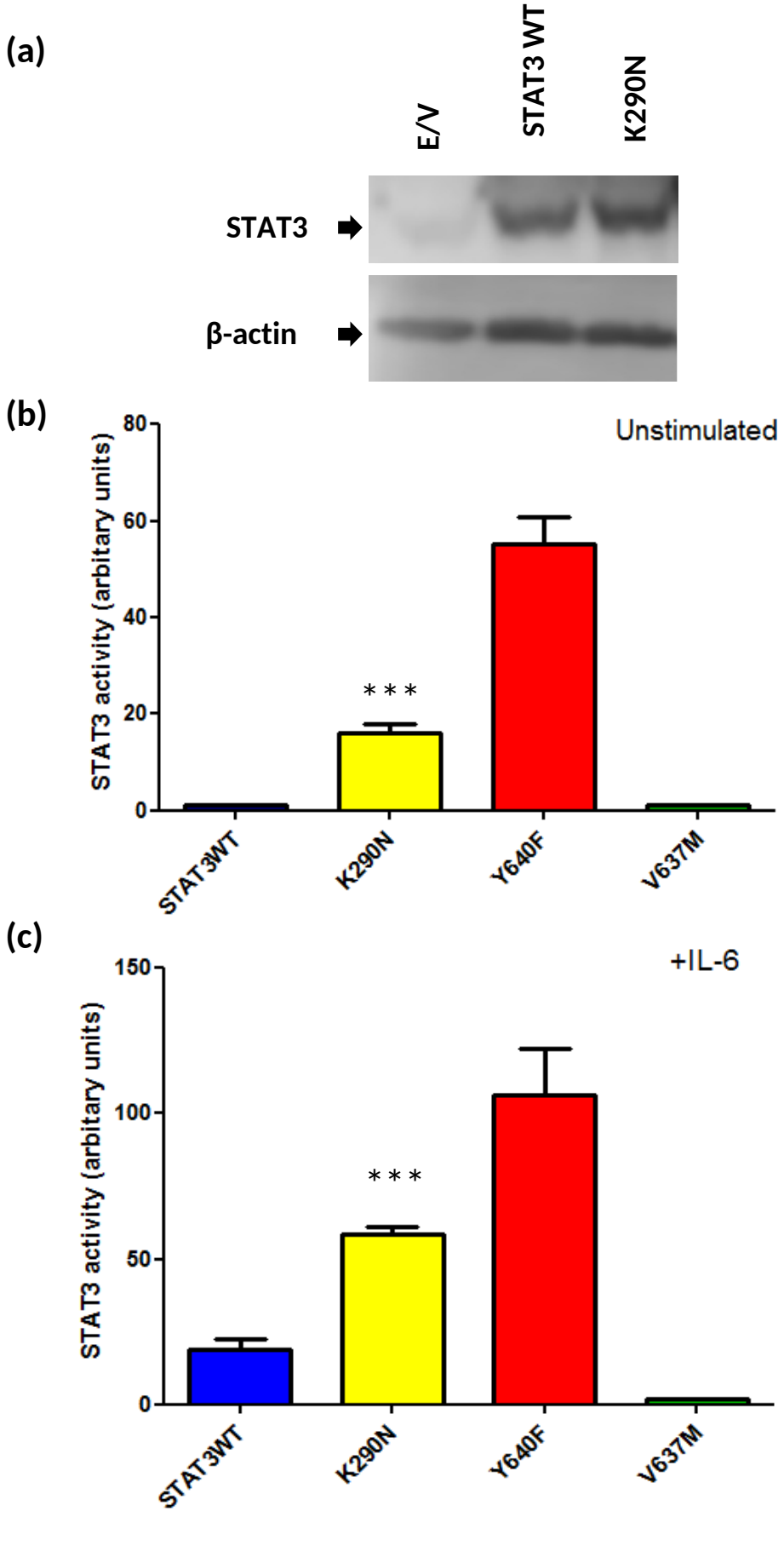

N-term
Coiled coil
DNA binding
linker
SH2
Trans-
activation
R103W
R246Q
F313L
K290N
Human kkleelqqkvsykg
Mouse kkleelqqkvsykg
Goat kkleelqqkvsykg
Zebrafish rkleelqqkvsykg

Fig 2


(a)
E/V
STAT3 WT
K290N
STAT3
β-actin
(b)
Unstimulated
STAT3 activity (arbitary units)
80
60
40
20
0
* * *
STAT3WT
K290N
Y640F
V637M
(c)
+IL-6
STAT3 activity (arbitary units)
150
100
50
0
* * *
STAT3WT
K290N
Y640F
V637M

Supplementary Table 1: Laboratory parameters

	Aged 14-20y	Aged 35-38y
Blood count Haemoglobin (NR 11.5-16.5g/dL)	8.2-13.0g/dL	9.4-10.5g/dL (↓)
Neutrophils (NR 2-7.5x10^9/L)	2.3-9.4x10^9/L	7.9-11.8x10^9/L (↑)
Lymphocytes (NR 1.5-4x10^9/L)	2.0-6.7x10^9/L	2.0-9.5x10^9/L
CD3+ cells (NR 918-2023x10^6/L)	3601-4526x10^6/L (↑)	1944-8927x10^6/L (↑)
CD4+/8+ cell ratio (NR 0.9-1.9)	0.55 (↓)	0.47-0.8 (↓)
CD19+ cells* (NR 42-461x10^6/L)	574-552x10^6/L (↑)	<1-48x10^6/L (↓)
CD56+ cells (NR 41-339x10^6/L)	96x10^6/L	77-278x10^6/L
Platelets (NR 150-400x10^9/L)	9-12x10^9/L (↓)	257-345x10^9/L
C-reactive protein (NR<5)	30-64mg/L (↑)	44-116mg/L (↑)
Creatinine (NR<90)	142-168 µmol/L(↑)	231-306** µmol/L(↑)
Liver function Aspartate aminotransferase (AST) (NR 13-35U/L)	16-23 U/L	14–18 U/L
Alanine aminotransferase (ALT) (NR 7-35 U/L)	11-29 U/L	7-8 U/L
Alkaline phosphatase (AP) (NR 30-130 U/L)	111-183 U/L	75-505 U/L
Gamma-glutamyl transferase (GGT) (NR 5-39 U/L)	96-158U/L (↑)	164-464 U/L (↑)
Immunoglobulins		
IgG (NR 7-16g/L)	4.5g/L(↓)	10-13g/L***
IgA (NR 0.8-4g/L)	0.3-0.4g/L (↓)	0.05-0.11g/L (↓)
IgM (NR 0.4-2g/L)	0.8-0.9g/L	0.45- 0.63g/L
Vaccine Responses	N/A	N/A

*switched memory B cells not tested, **dialysis-dependent from age 3, ***post-immunoglobulin replacement. NR=normal range

Supplementary Table 2. Histological findings

Tissue	Age at sampling	Report
Spleen	13 years	Lymphocytosis of red pulp Focal aggregates of foamy macrophages
Liver	28 years	Prominent lymphoid aggregates centred around the bile ducts with moderate fibrous expansion, focal bridging necrosis and some ductopenia. Mild interface hepatitis and sinusoidal dilatation in hepatic lobules, with foci of hepatocyte dropout and associated chronic inflammation.
Duodenum	34 years	Almost devoid of surface glandular epithelium. Intact villi are of normal architecture with no villous blunting or increase in intraepithelial lymphocytes. No nodular lymphoid hyperplasia, granuloma, active inflammation or ulceration. No giardia, cryptosporidia or CMV. Absent CD138+ plasma cells within lamina propria.
Termimal ileum	34 years	Mild villous distortion, oedema and patchy neutrophilic infiltrate within the lamina propria with focal cryptitis. Absent plasma cells. No granuloma formation. No luminal organisms or CMV-like viral inclusions.
Colon	35 years	Preserved crypt architecture showing paucicellular lamina propria with no plasma cells. No active inflammation or granulomata.
Bone Marrow	35 years	Hypocellular. Three haemopoietic series identified with maturation. Few Megakaryocytes and occasional T lymphocytes present. No dysplastic features, fibrosis or granulomas. Flow cytometry: Reversed CD4:CD8 ratio. High percentage of LGLs (large granular lymphocytes). Fifteen percent of all white blood cells are CD14+ CD11B+ monocytes. Absent B-cells. No obvious infiltrate.
Peripheral blood	35 years	Flow cytometry: Reversed CD4:CD8 ratio. Increased CD57+ CD8+ population. Absent B-cells.