Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations


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Key Points:

- Germline, gain-of-function mutations in *STAT3* lead to lymphoproliferation and autoimmunity with prominent cytopenias.
- Mutations in *STAT3* cause altered regulatory T cells and cytokine signaling.
Germline, loss-of-function mutations in the transcription factor \( STAT3 \) cause immunodeficiency, while somatic, gain-of-function mutations in \( STAT3 \) are associated with large granular lymphocytic leukemic, myelodysplastic syndrome, and aplastic anemia. Recently, germline mutations in \( STAT3 \) have also been associated with autoimmune disease. Here we report thirteen individuals from ten families with lymphoproliferation and early-onset, solid organ autoimmunity associated with nine different germline, heterozygous mutations in \( STAT3 \). Patients exhibited a variety of clinical features, with most having lymphadenopathy, autoimmune cytopenias, multi-organ autoimmunity (lung, gastrointestinal, hepatic, and/or endocrine dysfunction), infections and short stature. Functional analyses demonstrate that these mutations confer a gain-of-function in \( STAT3 \) leading to secondary defects in STAT5 and STAT1 phosphorylation and the regulatory T cell compartment. Treatment targeting a cytokine pathway that signals through STAT3 lead to clinical improvement in one patient, suggesting a potential therapeutic option for such patients. These results suggest that there is a broad range of autoimmunity caused by germline \( STAT3 \) gain-of-function mutations, and that hematologic autoimmunity is a major component of this newly-described disorder. Some patients for this study were enrolled in a trial registered at ClinicalTrials.gov #NCT00001350.
Introduction

The control of lymphocyte proliferation and tolerance are essential for both host defense and protection against self-directed immune attack. The discovery of autoimmune disorders with Mendelian inheritance that lead to a loss of tolerance has provided critical insights into biologic pathways important for regulation of the human immune response. For example, disorders of Fas-mediated apoptosis perturb peripheral lymphocyte tolerance and lead to the lymphoproliferation and hematologic autoimmunity seen in autoimmune lymphoproliferative syndrome (ALPS).\(^1,2\) Similarly, mutations in genes important for the development and/or function of regulatory T cells dramatically undermine peripheral tolerance, producing early-onset, polyendocrine autoimmunity and immune dysregulation as seen in immunodeficiency polyendocrinopathy enteropathy x-linked (IPEX) and IPEX-like disorders.\(^3\) Mutations in AIRE, important for central T cell tolerance, lead to a less severe syndrome of multiendocrine autoimmunity and mucocutaneous candidiasis called autoimmune polyendocrinopathy candidiasis and ectodermal dysplasia (APECED).\(^4,5\) An improved understanding of the mechanisms which regulate immune tolerance has the potential to improve the treatment of common autoimmune disorders by identifying and then targeting the relevant cellular pathways.

To further this process of discovery, we performed whole exome sequencing of DNA from patients with early-onset autoimmunity and lymphoproliferative disease reminiscent of ALPS without a known genetic cause. We identified heterozygous STAT3 mutations in thirteen individuals from ten families, consistent with an apparent autosomal dominant inheritance in certain kindreds and sporadic occurrences in others. Recently, a series of five patients with germline mutations in STAT3 associated
predominantly with neonatal diabetes was reported by Flanagan et al., consistent with the hypothesis that such mutations can cause autoimmune disease. Here we present genetic and functional data that reveal dysregulated cytokine signaling due to gain-of-function STAT3 mutations with important implications for understanding lymphocyte tolerance and the pathogenesis of systemic autoimmunity.
Methods

Patients

Participants were enrolled independently at each institution into research studies utilizing whole exome sequencing to identify genetic causes of autoimmunity. Written and informed consent was obtained for living patients and their family members. These studies were approved by the Institutional Review Boards of the authors’ institutions. Patients 2-4 and Family 1 were enrolled in ClinicalTrials.gov identifier NCT00001350.

Whole exome sequencing

Genomic DNA was extracted from whole blood or saliva and coding regions enriched (SureSelect All Exon, Agilent Technologies, Santa Clara, CA or as described for Family 2) followed by high-throughput next generation sequencing (Illumina, San Diego, CA) at individual institutions. Following quality control, alignment to GRCH37 or GRCH38, and variant calling, exome data were analyzed using a variety of institution-specific pipelines to identify novel or rare variants, including de novo variants, and cross-referenced between patients to focus on candidate genes. Putative disease-causing STAT3 variants were confirmed by dideoxy-based sequencing of PCR amplicons in each proband, and relevant family members where available. The STAT3 mutation in Patient 6 was detected by targeted dideoxy-based sequencing of the coding regions of STAT3 (Correlagen, Westborough, MA). Subsequent whole exome sequencing of this patient’s DNA did not identify another candidate disease-causing variant.

Expression plasmids
Expression plasmids with the observed mutations were generated using a plasmid containing the wild-type (WT) STAT3 cDNA (NM_139276, Origene, Rockville, MD) that was mutated with QuikChange site-directed mutagenesis (Agilent Technologies). Primers used are reported in Table S1. Targeted sequences were confirmed by dideoxy-based sequencing. Plasmids expressing a loss-of-function STAT3 mutation (p.K658E, LOF) observed in hyper-IgE patients (personal communication by Dr. S. Holland) and a gain-of-function STAT3 mutation (p.N647I, GOF) observed in large granular lymphocytic leukemia were provided by Dr. S. Holland and generated from the same WT plasmid.

**Transfection experiments**

WT, GOF, LOF, or mutant STAT3 plasmids were transfected into the STAT3-deficient A4 human colon cancer cell line (derived from DLD cells, kindly provided by Dr. G. Stark) by transient transfection (Lipofectamine, Life Technologies, Carlsbad, CA). The parental DLD cell line was used as a control for some assays as shown. Total STAT3 protein and phospho-STAT3 (Y705) were measured by Western blot (clones 79D7 and D3A7 respectively, Cell Signaling Technology, Danvers, MA) with or without simulation with IL-6 (10ng/mL, Miltenyi Biotec) for 15min. To confirm expression of mutant A703T, total STAT3 was detected using clone 124H6 (Cell Signaling Technology). For STAT3 luciferase studies, cells were co-transfected with 250ng of the indicated STAT3 plasmid and a STAT3-responsive firefly luciferase reporter mixed with a control renilla plasmid (CMV promoter) per manufacturer’s instructions (Cignal STAT3, Qiagen, Germantown, MD), followed by measurement of firefly and renilla luciferase (Dual-Luciferase Reporter...
Assay, Promega, Fitchburg, WI). No change in expression of renilla luciferase was seen when cells were transfected with WT, LOF, or GOF STAT3 (data not shown). In some experiments, cells were stimulated with recombinant human IL-6 (10ng/mL) or IFN-α (50ng/mL, both Miltenyi Biotec, San Diego, CA) for 12h. Results represent the ratio of reporter (firefly) to control (renilla) luciferase or are normalized as fold-change in the ratio as compared to WT plasmid. Cytokine stimulation was conducted in serum containing media.

Detection of T cell subsets

Flow cytometry was conducted on patient samples at four separate facilities. Typically, PBMCs were isolated by density centrifugation and resuspended in RPMI1640 supplemented with penicillin, streptomycin and L-glutamine along with 10% FCS. Double-negative T cells were measured by flow cytometric analysis of PBMCs from patients at individual institutions and results represent the percentage of CD4\textsuperscript{neg}CD8\textsuperscript{neg} T cells in the CD3\textsuperscript{+}TCRα/β\textsuperscript{+} T cell subset. For TH17 cells, PBMCs were co-stimulated with PMA and calcimycin or ionomycin for between 4.5h and overnight and flow cytometric analysis of intracellular IL-17 performed after fixation and permeabilization. Results shown represent healthy controls and patients performed in parallel at different institutions.

Regulatory T cell (Treg) analysis was performed at individual institutions. For Patients 2, 3, 4 and 9, PBMCs were first stained with Live/Dead Fixable Aqua viability dye (Invitrogen, Carlsbad, CA), CD3 and CD25 (both BD Biosciences, San Jose, CA). Cells were then fixed and permeabilized with a FOXP3 staining kit per manufacturer's
instructions (eBioscience, San Diego, CA). Cells were next stained with FOXP3 (clone 236A/E7, eBioscience) and CD4 (BD Biosciences). Tregs from Patient 1 were assayed in the Clinical Laboratory Improvement Amendments-certified Clinical Immunodiagnostic and Research Laboratory of the Medical College of Wisconsin and fresh cells were stained for cell surface CD4 and CD127 and intracellular FOXP3. For patient 6, cells were washed and stained with a viability dye to exclude dead cells (Ghost Viability dye, Tonbo, San Diego, CA). Cells were next surface stained with CD4 (eBioscience) and CD25 (BD Biosciences), and then fixed and permeabilized using a FOXP3 staining kit per manufacturer’s instructions (eBioscience). Cells were then stained with intracellular antibodies CD3 (Biolegend, San Diego, CA) and FOXP3 (clone 259D/C7, BD Biosciences) for 1h before washing and acquisition. Data was analyzed with FlowJo (Ashland, OR) software.

**EBV-transformed cell lines and phosphoflow**

EBV-transformed lymphoblastoid cell lines were generated from available patients (Patients 2, 3, 4, 5, and 9) and healthy controls by standard methods. PBMCs were isolated by density centrifugation and resuspended in RPMI1640 supplemented with penicillin, streptomycin and L-glutamine along with 10% FCS. For phosphoflow for Patients 2, 3, 4 and 9, cells were rested in serum-free RPMI1640 prior to cytokine stimulation. Cytokine stimulations were then performed in serum-free RPMI1640 for 20min at 37°C with IL-2 (80U/mL), IL-21 (100ng/mL), IL-27 (75ng/mL) or IFN-γ (500ng/mL). After stimulation, cells were fixed in 1.6% paraformaldehyde for 10min at room temperature and then resuspended in 100% cold methanol and incubated
overnight at -20°C. Cells were then washed and stained with fluorochrome-conjugated phospho-STAT antibodies (BD Biosciences). For phosphoflow for Patient 1, cells were stimulated with IL-6 (10ng/mL) or IL-21 (5ng/mL) for the indicated times. Cells were then fixed with BD Cytofix and permeabilized with BD Phosphoflow Perm Buffer III (both BD Biosciences) per manufacturer’s instructions. Cells were incubated with phospho-STAT3 antibodies at room temperature followed by flow cytometric analysis. For phosphoflow for Patient 5, cells were stimulated with IL-2 (1000U/mL) or IFN-γ (1000ng/mL) at 37°C for times indicated. Cells were then washed, fixed with BD Cytofix and permeabilized with BD Phosphoflow Perm Buffer III. After washing, cells were incubated with phospho-STAT antibodies at 4°C followed by flow cytometric analysis.

Data was analyzed with FlowJo software.

**Real-time quantitative RT-PCR**

SOCS3 transcript was measured by real-time quantitative RT-PCR in EBV-transformed patient-derived cell lines. Cells were serum starved followed by 16h incubation in RPMI1640 + 2% FCS with or without IL-21 (50ng/mL). Transcript levels of SOCS3 were normalized to endogenous β-actin control (both Taqman primer/probe sets were purchased from Life Technologies, Grand Island, NY). Results represent fold-change compared to an unstimulated healthy control.

**Statistics**

Results were analyzed with Mann-Whitney or unpaired two-tailed Student t test using Graph-pad Prism software and \( P < .05 \) considered statistically significant.
Results

Whole exome sequencing identifies \textit{STAT3} variants in patients with lymphoproliferation and early-onset autoimmune disease.

Whole exome sequencing was performed at multiple centers on a cohort of patients with prominent lymphoproliferation, including lymphadenopathy and/or hepatosplenomegaly, as well as early-onset, multi-system autoimmunity for whom a genetic diagnosis had not been established (Table 1). Upon comparing results in a subset of the cohort, it was noted that mutations in \textit{STAT3} were observed in thirteen patients from ten families (Tables 1 and S2). All were missense variants that were predicted to be deleterious by a variety of algorithms (Table S2) and absent from public databases of genetic variation (dbSNP and COSMIC), apart from c.1243G>A (p.E415K), which was recently reported as a somatic variant in liver cancer (COSMIC database). In contrast to the somatic, gain-of-function mutations seen in malignancy which cluster in the SH2 domain,\textsuperscript{8} the germline mutations identified here were located in multiple domains including the all-alpha, DNA binding, SH2, and C-terminal transactivation domains (Figure 1A). A single variant, c.2147C>T (p.T716M), was observed in three individuals with autoimmune enteropathy, two of whom were related (Family 2, previously reported\textsuperscript{7}). Interestingly, this same mutation was observed in a patient with early-onset type I diabetes.\textsuperscript{6}

In contrast to the recently reported patients with \textit{STAT3} mutations who predominantly had type I diabetes and enteropathy,\textsuperscript{6} within our cohort hematologic autoimmunity was most prevalent including autoimmune hemolytic anemia,
neutropenia, and/or thrombocytopenia (Table 1). Similar solid organ autoimmunity was present in some of our patients, including autoimmune enteropathy (Table S3). Others also exhibited arthritis, lung disease consistent with lymphocytic interstitial pneumonia, hepatitis, atopic dermatitis, alopecia and/or scleroderma.

Several patients also had recurrent and/or severe infections (Table 2), which was not a prominent feature of the five patients recently reported.6 We noted four patients with fungal infections. Whether this particular finding may become more common as more patients are described with this disorder remains to be seen. In several of the patients with recurrent infections, hypogammaglobulinemia was noted (Table 2) but only Patient 4 fulfilled criteria for common variable immune deficiency (Table S4). Of note, the chronic immune suppression required in these patients has the potential to cause some, but not all of these phenotypes.

Similar to the previously reported patients6, we noted post-natal short stature <5% percentile in a majority of our patients (7/13 patients). Several patients exhibited profound growth failure as represented by their growth curves (Figure S1A-D). The growth hormone axis was assessed in three patients; two patients were treated with growth hormone with good response (Table S5).

Additional immunologic laboratory features among our cohort were variable but included moderate T cell lymphopenia, hypogammaglobulinemia, and elevated double negative (CD4negCD8neg) CD3+TCRα/β+ T cells (DNTs) in many patients (Table 2). The observation of elevated DNTs is a consistent feature of ALPS and FAS pathway defects, but has also been observed in pediatric patients with more common autoimmune diseases and likely reflects a lymphoproliferative state.1,2,10 FAS-mediated
apoptosis was assayed in five patients, one of whom showed a defect (Tables 2 and S6). Targeted sequencing in this patient excluded defects associated with classic ALPS.

In two families (Patient 4 and Family 1), there were family members carrying a STAT3 mutation who had a less severe phenotype or were asymptomatic (father of Patient 4, not included here). In Family 1, Patient 11 experienced a single, childhood episode of lymphoproliferation and hematologic autoimmunity which resolved without sequelae. A third sibling in Family 1 also carries the STAT3 mutation but is asymptomatic. Therefore, not only is the clinical phenotype of these patients diverse as described above, there appear to be carriers of these mutations who display incomplete penetrance.

The observed STAT3 mutations lead to enhanced transcriptional activity but not constitutive phosphorylation of STAT3.

STAT3 encodes signal transducer and activator of transcription 3, one of a family of transcription factors that play critical roles in affecting cytokine-induced changes in gene expression.11-13 Following receptor ligation, JAK kinases recruit, phosphorylate, and activate STATs. STATs then translocate to the nucleus and interact with specific DNA elements as part of transcriptional regulatory complexes, which, depending on the cytokine and STAT molecule involved, can promote both pro- and anti-inflammatory pathways.

The stereotyped clinical phenotype of our patient cohort with multisystem autoimmunity and lymphoproliferation as described above and further in Table S7 was quite distinct from that associated with germline STAT3 mutations shown to confer a
loss-of-function (i.e., hyper-IgE syndrome).\textsuperscript{14,15} Analogous to the contrasting allelic disorders caused by gain- and loss-of-function mutations in $\text{STAT1}$,\textsuperscript{13,16,17} we hypothesized that the changes observed here in $\text{STAT3}$ lead to a gain-of-function. Mechanistically, gain-of-function $\text{STAT3}$ mutations could be predicted to lead to autoimmunity because of the known involvement of STAT3 in intracellular signaling pathways relevant to immune dysregulation, including proinflammatory signaling and inhibition of Treg and enhancement of Th17 cell fate determination.\textsuperscript{18-21} Furthermore, somatic, gain-of-function $\text{STAT3}$ mutations are reported in association with large granular lymphocytic leukemias and acquired aplastic anemia, both of which involve a prominent autoimmune component.\textsuperscript{8,22} Indeed, the recent series of five patients with autoimmunity and $\text{STAT3}$ mutations included evidence that the mutations conferred a gain-of-function.\textsuperscript{6}

To test the effect of the mutations found in our cohort on STAT3 function, constructs containing the variants were transfected into a $\text{STAT3}$-deficient cell line (A4 cells).\textsuperscript{9} Each variant produced detectable immunoreactivity via Western blot (Figure 1B), however the p.A703T mutation was detectable only with a different anti-STAT3 antibody presumably due to a loss of the antibody binding site in the presence of the mutation (Figures 1B and S2). $\text{STAT3}$ was not significantly phosphorylated at baseline in any mutant, but was phosphorylated normally in response to IL-6 stimulation (Figure 1B). At baseline, all mutants with the exception of p.V353F conferred significantly increased transcriptional activity compared to WT (Figure 1C). The cytokines IL-6 and IFN-$\alpha$, both activators of $\text{STAT3}$, were used to stimulate the transfected cells. Statistically
significant increases in \textit{STAT3} activity compared to WT were seen for each mutant with either one or the other cytokine and typically for both (Figure 1D).

A major down-stream target of STAT3 is suppressor of cytokine signaling 3 (SOCS3). Transcript levels of \textit{SOCS3} were elevated both at baseline and after IL-21 stimulation in EBV-transformed cell lines from four patients (Figure 2), including for mutant p.V353F, which did not have baseline activity higher than WT by luciferase. This finding highlights differences in these two assays that are measuring different outcomes, transfection and expression in a \textit{STAT3}-deficient cell line and endogenous expression from a patient-derived cell line. It is possible that despite serum starvation prior to the outset of the experiments, there is still residual STAT3 agonist signals present in the STAT3 mutant cell line leading to a change in baseline activity. In combination, the findings in Figures 1 and 2 provide strong evidence that the observed mutations result in a gain of STAT3-mediated transcriptional function at baseline and/or with cytokine activation.

The effects of gain-of-function \textit{STAT3} mutations on the phosphorylation of STAT3 in patients with autoimmune disease are unknown.\textsuperscript{6} Our transfection experiments suggested there was no constitutive phosphorylation of STAT3. To examine the effects of gain-of-function \textit{STAT3} mutations on STAT3 phosphorylation in lymphocytes, we used EBV-transformed cell lines from four patients bearing different \textit{STAT3} mutations. Cell lines allowed us to minimize the variability of cellular activation state and the effects of concurrent treatment with immunosuppressants in primary patient samples. Similar to transfected cells, there was no phosphorylation of STAT3 at baseline, and phosphorylation of STAT3 in response to IL-21 was normal in all four
patient-derived cell lines as compared to cell lines derived from healthy controls (Figure S3A). This finding was corroborated in primary cells from Patient 1 (Figure S3B).

**Impaired STAT5 and STAT1 phosphorylation with gain-of-function STAT3 mutations.**

Our patients shared multiple clinical characteristics with STAT5b deficient patients. Since phosphorylation of several STATs, including STAT5, can be negatively regulated by the STAT3-target SOCS3, we examined cytokine-induced phosphorylation of STAT5 in patient-derived EBV-transformed cell lines. In response to IL-2, STAT5 phosphorylation was lower than healthy controls (Figure 3A), suggesting that STAT3 gain-of-function diminishes the STAT5 response. This was also found in primary cells from available patients (Figure S4). Phosphorylation of STAT5 remained decreased over the examined time course (Figure S5A). Similarly, STAT1 phosphorylation after IFN-γ was decreased in patient-derived cell lines across time points (Figure 3B and Figure S5B), as well as in available primary cells (Figure S6). This data suggests that upregulation of STAT3 transcriptional activity may have consequences for other cytokine signaling pathways.

**Impaired T regulatory cell compartment in patients with gain-of-function STAT3 mutations.**

STAT3 is known to play an important role in influencing T cell lineage decisions as they integrate environmental cues. Specifically, SOCS3, which is upregulated by STAT3, can suppress Treg function. The Treg compartment in two of the recently described
patients with gain-of-function mutations in STAT3 was reportedly reduced.\textsuperscript{6} Analysis of peripheral blood cells from our patients within the same institution (Figure 4A) showed diminished numbers of Treg cells as well as decreased expression of CD25, which is required for Treg function (Figure 4B&C). Two additional patients examined at other institutions also exhibited decreased expression of FOXP3 and/or CD25 (Figure S7).

**Peripheral blood mononuclear cells from a patient with a gain-of-function STAT3 mutation exhibit delayed kinetics of de-phosphorylation.**

The mechanism by which STAT3 gain-of-function produces downstream effects such as increased SOCS3 transcription could not be explained by hyper-phosphorylation (Figure S3), as has been reported for gain-of-function mutations in STAT1.\textsuperscript{17} However, after stimulation with IL-6, PBMCs from Patient 1 exhibited delayed de-phosphorylation of pSTAT3 compared to healthy controls (Figure S8). This may provide an alternative explanation for increased STAT3 activity in some of these patients.

**Two patients with germline, gain-of-function STAT3 mutations received hematopoietic stem cell transplants.**

All of the symptomatic patients reported here required significant immunosuppression to control their autoimmune diseases and two patients (Patients 7 and 8) underwent hematopoietic stem cell transplantation (HSCT) for refractory autoimmunity as detailed in Table S8. Patient 8 died 138 days out from transplant of severe graft versus host disease with disseminated adenoviral infection. Patient 7 is alive and well with
improved growth and complete remission of her autoimmune disease, and in particular she has no signs of the previously severe autoimmune enteropathy.

**Blockade of the IL-6 pathway in a patient with a gain-of-function STAT3 mutation resulted in clinical improvement and decreased Th17 cells.**

Over the span of four years, the arthritis and scleroderma-like skin changes in Patient 1 failed to respond to treatment with anti-TNFα, anti-IL-1 and anti-B cell therapies, although his severe autoimmune hemolytic anemia responded well to rituximab. IL-6 is one of the primary cytokines that utilizes STAT3 for signal transduction. When considering additional therapeutic options for this patient after discovering his gain-of-function STAT3 mutation, we hypothesized that IL-6 blockade might ameliorate his disease and so he was treated with the anti-IL6R monoclonal antibody therapy tocilizumab. Within months of starting tocilizumab, he exhibited a dramatic improvement of previously fixed flexion contractures of his distal interphalangeal joints (Figure 5). Similarly, his inability to fully open his hands due to a combination of metacarpal-phalangeal joint contractures and skin tightening also improved as measured by two pediatric rheumatologists. In keeping with the expected outcome of modulating STAT3 activity with treatment, Th17 cells were initially elevated in this patient and normalized after anti-IL6 treatment (Figure S9). Th17 frequencies were measured in six other patients but did not appear to be elevated (Figure S9), although concurrent immunosuppressive regimens preclude definitive conclusions regarding the potential role of Th17 cells in this patient cohort.
Discussion

Here we describe gain-of-function STAT3 mutations in a cohort of patients with lymphoproliferation and childhood-onset autoimmunity. We demonstrate that these mutations confer increased STAT3 transcriptional activity, impair cytokine signaling via other STAT molecules, and diminish the Treg compartment.

Our findings further support a role for human STAT3 in immune regulation and extend the spectrum of STAT3-associated disease. Patients bearing germline, loss-of-function STAT3 mutations have immunodeficiency and exhibit infections quite distinct from those observed in this cohort, along with impaired Th17 differentiation, markedly higher levels of IgE, and far more common connective tissue abnormalities. Somatic, gain-of-function mutations in STAT3 are associated with lymphoproliferative neoplasms, acquired aplastic anemia, and myelodysplastic syndrome, but again, appear to be distinct from the phenotype in this cohort, especially given the relative dearth of neoplastic disease among these patients with congenital gain-of-function mutations.

While this cohort shares common clinical characteristics, including autoimmune cytopenias, lymphoproliferation, and short stature, affected individuals varied in their age at presentation and extent of solid organ autoimmunity. Four of the five patients recently reported by Flanagan et al. with early-onset autoimmunity and STAT3 mutations had neonatal diabetes. Among them was a young patient with type I diabetes and autoimmune enteropathy bearing the same c.2147C>T mutation (p.T716M) observed in Patient 7 and Family 2. Although our patients with p.T716M mutations did not have type I diabetes, two of our cohort with other STAT3 mutations...
did. This partial overlap in phenotype suggests that germline, gain-of-function mutations in STAT3 produce a strong susceptibility to immune dysregulation. However, the specific target organs involved can differ even between patients with the same mutation, similar to other monogenic disorders of autoimmunity.5

In addition to an ALPS-like phenotype, patients in our cohort share many clinical features of patients with STAT5b deficiency. Mutations in STAT5b can lead to endocrine abnormalities, short stature, recurrent infection, eczema, autoimmunity, and lymphocytic interstitial pneumonia.27 This is presumably due, in part, to defects in responses to growth hormone and in Treg number and function, both of which depend upon normal STAT5b signaling.27,28 Further, the immune dysregulatory phenotype seen in some of our cohort resembles that of patients with CD25 or FOXP3 mutations leading to the loss of Tregs.3 As we observed defects in both pSTAT5 responses and the Treg compartment in our patients, we hypothesize that a possible mechanism for autoimmunity in STAT3 gain-of-function is impaired absolute Treg function as a result of diminished STAT5 signaling, possibly related to increased SOCS3 activity. An absolute reduction in function could be reflective of diminished numbers of Tregs as we show. This could also be related to a defect in Treg function, which we are not able to assess due to leukopenia and decreased surface expression of CD25, which have precluded sorting the CD25+ Treg population in our patients.

The mechanism underlying the gain-of-function in STAT3 has not yet been elucidated, but we provide evidence for at least one potential mechanism by showing delayed phosphorylation kinetics after stimulation of primary patient cells from Patient 1 with IL-6. This may be the result of enhanced DNA binding, this mutation is in the DNA
binding domain, leading to prolonged nuclear retention of STAT3. It is probable that the mechanism of gain-of-function STAT3 mutations will be different depending upon the particular mutation and protein domain affected, similar to what has been reported with loss-of-function STAT3 mutations.29

Two patients with gain-of-function STAT3 mutations received HSCT for their refractory autoimmunity, and one of those patients had complete remission of her autoimmune disease. Due to the low numbers of transplanted patients it is not possible to give an estimate of the likelihood of success of HSCT in this condition. However, sixteen children with similarly severe and complex autoimmune disease have also undergone HSCT in Newcastle; fourteen are alive and twelve are in complete remission from their disease (personal communication, A.J.C.). These data suggest that replacement of the hematopoietic compartment may alleviate tissue-specific autoimmune disease.

One of the patients here was treated with anti-IL6R monoclonal antibody therapy with significant improvement of his otherwise refractory polyarthritis and skin disease. While this observation was made in a single patient, this patient’s response suggests that treatment of other patients with STAT3-associated autoimmunity with therapies targeting the STAT3 pathway is worth investigating. In addition to inhibitors of the IL-6 pathway, small molecule inhibitors of STAT3 are under clinical investigation for malignancy.30,31

In summary, we describe a relatively large cohort of patients with a monogenic disorder of early-onset autoimmunity and lymphoproliferation caused by gain-of-function, germline STAT3 mutations. These findings significantly expand the clinical
spectrum of this newly-described monogenic disorder and provide evidence for a potential mechanism whereby this gain-of-function leads to altered immune cell tolerance via interference with the activation of other STAT molecules. These findings suggest a broad range of autoimmune and lymphoproliferative disorders may be explained by inborn errors of cytokine signaling. They also provide a new model for studying the biology and clinical consequences of the complex regulatory networks involved in STAT-mediated signaling. Furthermore, we provide a rationale for the use of inhibitors of this pathway, including anti-IL-6R therapy as shown here, or, potentially, small molecule inhibitors of STAT3, to achieve substantial therapeutic benefit in this and related conditions.
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Conflict-of-interest disclosure

The authors declare no competing financial interests.
REFERENCES


Table 1. Patient characteristics.

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<td>7y, M</td>
<td>31y</td>
<td>p.T663I</td>
<td>AIHA, AITP</td>
<td>No</td>
</tr>
<tr>
<td>Patient 3</td>
<td>3y, M</td>
<td>25y</td>
<td>p.R152W</td>
<td>AIHA, AITP</td>
<td>IDDM</td>
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<tr>
<td>Patient 4</td>
<td>13y, M</td>
<td>32y</td>
<td>p.V353F</td>
<td>AIHA, AITP, AIN</td>
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<tr>
<td>Patient 5</td>
<td>3y, F</td>
<td>5y</td>
<td>p.Q344H</td>
<td>AIHA</td>
<td>No</td>
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<tr>
<td>Patient 6</td>
<td>5y, F</td>
<td>9y</td>
<td>p.E415K</td>
<td>none</td>
<td>IDDM</td>
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<tr>
<td>Patient 7</td>
<td>&lt;1y, F</td>
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<td>p.T716M</td>
<td>AIHA, AITP, AIN</td>
<td>Hypothyroid</td>
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<tr>
<td>Patient 8</td>
<td>3y, F</td>
<td>Dec 11y</td>
<td>p.N420K</td>
<td>AIHA, AITP, AIN</td>
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*Family 1
<table>
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<tr>
<th>Patient 9</th>
<th>Proband</th>
<th>&lt;1y, F</th>
<th>26y</th>
<th>p.A703T</th>
<th>AIHA, AITP, AIN</th>
<th>No</th>
<th>Small bowel thickening</th>
<th>LIP, atopic dermatitis, alopecia</th>
<th>Yes</th>
<th>HSM</th>
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<tr>
<td>Patient 10</td>
<td>Father</td>
<td>15y, M</td>
<td>Dec 28y</td>
<td>p.A703T</td>
<td>AIHA, AIN</td>
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<td>No</td>
<td>LIP</td>
<td>Yes</td>
<td>HSM</td>
<td>n/a</td>
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<tr>
<td>Patient 11</td>
<td>Sibling</td>
<td>12y, F</td>
<td>24y</td>
<td>p.A703T</td>
<td>AITP, AIN</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>HSM</td>
<td>n/a</td>
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<tr>
<td>Patient 12</td>
<td>Proband</td>
<td>&lt;1y, M</td>
<td>4y</td>
<td>p.T716M</td>
<td>none</td>
<td>No</td>
<td>Enteropathy</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 13</td>
<td>Father</td>
<td>EO, M</td>
<td>32y</td>
<td>p.T716M</td>
<td>AITP</td>
<td>No</td>
<td>Enteropathy</td>
<td>No</td>
<td>No</td>
<td>No</td>
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*Amino acid substitution based on coding sequence of NM_139276.2, †less than 5th%, ‡Percentile n/a.

Abbreviations: LAD indicates lymphadenopathy; EO, early childhood onset; Dec, deceased; AIHA, autoimmune hemolytic anemia; AITP, autoimmune thrombocytopenia; AIN, autoimmune neutropenia; IDDM, insulin dependent diabetes mellitus; LIP, lymphocytic interstitial pneumonia; HSM, hepatosplenomegaly; n/a, not available.
Table 2. Other immunologic laboratory parameters and infection history.

<table>
<thead>
<tr>
<th>Patient</th>
<th>STAT3</th>
<th>Hypogam</th>
<th>%DNTs</th>
<th>FAS-apoptosis defect</th>
<th>Other (ALC nadir/mm³)</th>
<th>Severe or recurrent infections</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>p.G421R</td>
<td>Yes</td>
<td>3.3%</td>
<td>n/a</td>
<td>T lymphopenia (208)</td>
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<tr>
<td>Patient 2</td>
<td>p.T663I</td>
<td>No</td>
<td>1.6-2.6%</td>
<td>No</td>
<td>(3100) Viral pneumonia, molluscum, ocular herpes</td>
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<tr>
<td>Patient 3</td>
<td>p.R152W</td>
<td>Yes*</td>
<td>2.7-3.5%</td>
<td>No</td>
<td>(1300) Recurrent herpes zoster</td>
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<tr>
<td>Patient 4</td>
<td>p.V353F</td>
<td>Yes</td>
<td>2.9%</td>
<td>No</td>
<td>B lymphopenia; poor memory B cells (710) Onychomycosis, skin HPV, herpes zoster, dermatophytic infection</td>
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<tr>
<td>Patient 5</td>
<td>p.Q344H</td>
<td>Yes</td>
<td>n/a</td>
<td>n/a</td>
<td>Disseminated Pseudomonas, Candida, and Serratia, UTI</td>
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<td>Patient 6</td>
<td>p.E415K</td>
<td>Transient</td>
<td>n/a</td>
<td>n/a</td>
<td>Neonatal sepsis, recurrent URI, esophageal candidiasis</td>
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<tr>
<td>Patient 7</td>
<td>p.T716M</td>
<td>No</td>
<td>&lt;1%</td>
<td>n/a</td>
<td>T lymphopenia (610) Lung infections</td>
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<tr>
<td>Patient 8</td>
<td>p.N420K</td>
<td>No</td>
<td>1.5%, 3.4%</td>
<td>No</td>
<td>T lymphopenia (913) Recurrent respiratory infections</td>
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<tr>
<td><strong>Family 1</strong></td>
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<tr>
<td>Patient 9 <strong>Proband</strong></td>
<td>p.A703T</td>
<td>No</td>
<td>1.4%</td>
<td>Yes</td>
<td>(2500)</td>
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<td>Patient 10 <strong>Father</strong></td>
<td>p.A703T</td>
<td>Yes</td>
<td>n/a</td>
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<td>Bacterial and fungal soft tissue, osteomyelitis</td>
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<td>Patient 11 <strong>Sibling</strong></td>
<td>p.A703T</td>
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<tr>
<td><strong>Family 2</strong></td>
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<tr>
<td>Patient 12 <strong>Proband</strong></td>
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<td>High B cells No</td>
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<td>Patient 13 <strong>Father</strong></td>
<td>p.T716M</td>
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<td>n/a</td>
<td>n/a</td>
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</table>

*Hypogammaglobulinemia after starting immunosuppression.
Abbreviations: Hypogam indicates hypogammaglobulinemia; n/a, not available; DNTs, double negative T cells; ALC; absolute lymphocyte count; HPV, human papillomavirus; UTI, urinary tract infection URI, upper respiratory infection.
FIGURE LEGENDS

Figure 1. **STAT3 mutations confer a gain-of-function.** (A) Schematic of human STAT3 protein showing the location of nine different STAT3 missense mutations and the resulting amino acid changes. Mutations were located throughout the protein in the all alpha, DNA binding, SH2, and C-terminal (transactivation) protein domains. The numbers represent the amino acid location based on STAT3 transcript variant 1 (protein model based on the Pfam protein family database). The p.T716M variant was detected in two different families (Patient 7 and Family 2). (B) Western blot of STAT3 expression. Wild-type (WT), gain-of-function (GOF), loss-of-function (LOF), and the nine different mutant STAT3 transcripts were expressed in the STAT3-deficient A4 cell line and expression of phosphorylated STAT3 (pSTAT3) and total STAT3 protein was determined using Western blot without (top) and with (bottom) IL-6 (15min, 10ng/mL). IL-6-treated DLD cells (parental A4 line) are shown as a control. Expression of all mutants led to the detection of STAT3 protein, with the exception of p.A703T, a mutation near the binding site of the antibody used here (clone 79D7). This variant was detectable after IL-6 activation and with a different antibody clone (Figure S2). Mutant STAT3 proteins were not phosphorylated at baseline, but were phosphorylated after stimulation with IL-6. (C&D) STAT3 binding activity as measured by luciferase assay. A4 cells were transfected with WT or the indicated STAT3 mutants and STAT3-driven firefly luciferase and control renilla reporters. (C) Luciferase was assayed at 48h and STAT3 activity is shown as a ratio of firefly/control for each construct. All constructs with the exception of V353F had significantly increased activity at baseline compared to WT. Results represent the mean +/-SEM of 5-10 independent experiments, the dotted
line represents WT (**P<.01, ***P<.001, ****P<.0001). (D) STAT3 activity following 12h activation with IL-6 (10ng/mL, white) or IFN-α (50ng/mL, black). All mutations demonstrated significantly increased STAT3 activity after at least one of the cytokine stimulations (P<.05), typically after both unless indicated (ns, not significant). Results shown represent fold-change versus WT for each cytokine stimulus; the dotted line represents a fold-change of 1 (no change from WT). Results represent the mean +/- SEM of 5 independent experiments.

Figure 2. Elevated expression of SOCS3 in patient-derived EBV-transformed cell lines. Expression of the STAT3-target SOCS3 was determined by quantitative RT-PCR in resting and IL-21-activated (50ng/mL for 21h) cell lines. Increased SOCS3 transcript was detected in patient-derived cells compared to healthy controls (HC1 and HC2). Results represent the fold-increased expression in SOCS3 transcript compared to unstimulated healthy control 1 (HC1) and are normalized to β-actin. Results represent the mean +/-SEM of 3 independent experiments. *P<.05; **P<.01; ***P<.001

Figure 3. Increased STAT3 activity in patient-derived EBV-transformed cell lines leads to decreased phosphorylation of other STAT molecules. (A) Impaired STAT5 phosphorylation in EBV-transformed cell lines derived from gain-of-function STAT3 patients. Cells from patients (red) and healthy controls (blue) were stimulated with IL-2 (80U/mL) and pSTAT5 measured by flow cytometry after 20min. Numbers indicate the difference in mean fluorescence intensity (MFI) of phospho-STAT5 obtained by subtracting the MFI of unstimulated from stimulated samples. Yellow lines represent
unstimulated patient samples and filled histograms unstimulated healthy control samples. (B) Impaired STAT1 phosphorylation in EBV-transformed cell lines derived from gain-of-function STAT3 patients. Cells from patients (red) and healthy controls (blue) were stimulated with IFN-γ (500ng/mL) and pSTAT1 measured by flow cytometry after 20min. Numbers indicate the difference in MFI of phospho-STAT1 obtained by subtracting the MFI of unstimulated from stimulated samples. Yellow lines represent unstimulated patient samples and filled histograms unstimulated healthy control samples.

Figure 4. STAT3 gain-of-function patients have decreased Treg numbers and functional markers. (A) Flow cytometric analysis of FOXP3+CD25+ Treg populations from Patient 4, gated on CD4+ T cells, compared to a healthy control. (B) Decreased Treg percentages in STAT3 gain-of-function patients. Treg populations were determined by flow cytometry. The percentages of CD4+ T cells that were FOXP3+CD25+ Tregs from patients were decreased compared to six healthy controls and two patients with ALPS who had FAS defects (*P<.05). (C) Treg cells from STAT3 gain-of-function patients have decreased expression of CD25. Treg populations (CD4+ FOXP3+CD127low) were determined by flow cytometry and the CD25 expression further analyzed by histogram. Tregs from patients demonstrate lower levels of CD25 expression than healthy controls.

Figure 5. Patient response to anti-IL6R monoclonal antibody therapy. Patient 1 received therapy with the anti-IL6R monoclonal antibody tocilizumab. He had notable
improvement of his long-standing polyarthritis and skin tightening as shown in the photographs. Indicated are the number of fixed DIP (distal interphalangeal) contractures of both his right and left hands at the designated times with respect to anti-IL-6R therapy. L, left; R, right; yr, year.
Figure 1

A. Schematic representation of STAT3 domains and mutations. The diagram shows the regions of interaction (P-P), all alpha, DNA binding, and SH2 domains, with specific mutations labeled (e.g., R152W, Q344H, E415K, G421R, T653I, V353F, T716M, N420K, A703T).

B. Immunoblot analysis of STAT3 phosphorylation (pSTAT3) and total STAT3 (STAT3) levels under different conditions (e.g., WT,mutants). The blots show the expression levels of STAT3 and pSTAT3 in response to stimuli (e.g., IL-6).

C. Bar graph representing the ratio of firefly/renilla luciferase activity (fold change in STAT3 activity relative to WT) for various conditions. The graph includes error bars and statistical significance indicators (e.g., ****, **, ns).

D. Line graph depicting the fold change in STAT3 activity (relative to WT) in response to different treatments (e.g., IL-6, IFNα). The graph includes error bars and statistical significance indicators (e.g., ****, **).
Figure 2

A. No Stimulation

STAT3 mutation

B. IL-21 Stimulation

STAT3 mutation
Figure 3

A. Patient 2  Patient 3  Patient 4  Patient 9

B. Patient 2  Patient 3  Patient 4  Patient 9

pSTAT5

pSTAT1
Figure 4

A. Healthy Control

Pt 4

FOXP3

CD25

B. Healthy Control

STAT3 GOF

C. Healthy Control

CD25
Figure 5

DIP Count

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<tr>
<th>Year</th>
<th>Right (R)</th>
<th>Left (L)</th>
</tr>
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<tbody>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Start</td>
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<tr>
<td>+1 yr</td>
<td>2</td>
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Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations