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**Complex Autoinflammatory Syndrome Unveils Fundamental Principles of JAK1 Kinase Transcriptional and Biochemical Function**

**Highlights**

- Janus kinase (JAK1) mutation underlies monogenic autoinflammatory disease
- S703I mutation enhances downstream signaling by transactivation of partnering JAKs
- Mosaicism and monoallelic expression shape JAK1 transcription patterns
- JAK inhibitor therapy resolves clinical disease

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**In Brief**

Monogenic errors of the Janus kinase (JAK) family, essential signal transduction hubs of the immune system, have dire consequences in immune function. Gruber et al. describe a JAK1 gain-of-function mutation with mosaicism and monoallelic expression that underlies a multi-system autoinflammatory disease, which is rescued by JAK inhibitor therapy.
Complex Autoinflammatory Syndrome Unveils Fundamental Principles of JAK1 Kinase Transcriptional and Biochemical Function

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SUMMARY

Autoinflammatory disease can result from monogenic errors of immunity. We describe a patient with early-onset multi-organ immune dysregulation resulting from a mosaic, gain-of-function mutation (S703I) in JAK1, encoding a kinase essential for signaling downstream of >25 cytokines. By custom single-cell RNA sequencing, we examine mosaicism with single-cell resolution. We find that JAK1 transcription was predominantly restricted to a single allele across different cells, introducing the concept of a mutational “transcriptotype” that differs from the genotype. Functionally, the mutation increases JAK1 activity and transactivates partnering JAKs, independent of its catalytic domain. S703I JAK1 is not only hypermorphic for cytokine signaling but also neomorphic, as it enables signaling cascades not canonically mediated by JAK1. Given these results, the patient was treated with tofacitinib, a JAK inhibitor, leading to the rapid resolution of clinical disease. These findings offer a platform for personalized medicine with the concurrent discovery of fundamental biological principles.

INTRODUCTION

Monogenic disease mutations afford the opportunity to study the bona fide function of human genes in vivo, which have guided our understanding of biology and medicine for decades. Undiagnosed disease programs, by means of next-generation sequencing, have recently provided a platform to identify, diagnose, and study these rare patients with unusual clinical presentations (Lee et al., 2014; Splinter et al., 2018; Yang et al., 2014). In turn, clinical management can, in some cases, be highly personalized.

To date, studies of rare immunologic diseases have identified germline gain-of-function (GoF) and loss-of-function (LoF) mutations throughout the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling axis (Dupuis et al., 2001; Etheridge et al., 2014; Flanagan et al., 2014; Hambleton et al., 2013; Holland et al., 2007; Kofoed et al., 2003; Macchi et al., 1995; Mead et al., 2012; Minegishi et al., 2006, 2007;
Russell et al., 1995; van de Veerdonk et al., 2011), the primary signal transduction pathway for cytokines. The Janus kinase (JAK) family contains four tyrosine kinases (JAK1, JAK2, JAK3, TYK2) constitutively associated with cytokine receptors. Upon cytokine binding, JAKs act in partnership to phosphorylate themselves, the receptors, and then STATs, which can then act directly as transcription factors or activate other signaling pathways further downstream (O’Shea et al., 2015). JAK1 is activated by a broad range of cytokines (γc, gp130, interferon [IFN], and interleukin-10 [IL-10] family cytokines). It can phosphorylate any signal transducer and activator of transcription (STAT) protein (STAT1–6) and is universally expressed in all tissues (O’Shea et al., 2015). Through the formation of specific combinations of cytokine receptors, JAK partners, and STAT dimers, JAK1 orchestrates unique downstream signals for each cytokine.

The need to better understand JAK regulation has deepened with the expanding clinical use of JAK inhibitors (O’Shea and Gadina, 2019). The breadth of successfully treated inflammatory conditions signifies the central pathophysiological role of JAK hyperactivity across immune diseases. However, the complete conditions signifies the central pathophysiological role of JAK hyperactivity across immune diseases. However, the complete list of disorders resulting from JAK-STAT dysregulation remains unknown. Furthermore, it is unclear which specific JAK-mediated pathways drive disease, a key issue for the design of inhibitors with greater selectivity for individual members of the JAK family.

Herein, we identify a mutation (S703I) of JAK1 in a patient with a severe, early-onset immunodysregulatory syndrome identified in our undiagnosed disease program. Using extensive next-generation genomic, molecular, and multi-parametric immunological tools, we probe the effects of S703I JAK1 in vitro and ex vivo to investigate clinical dysfunction in vivo.

RESULTS

Complex Immunodysregulatory Syndrome

We studied an 18-year-old female who was referred to our undiagnosed disease program with a complex primary autoimmune and atopic syndrome (Figure 1A). The patient was born to a non-consanguineous family with no history of immunologic disease. At birth, the patient was noted to have a widespread pustular rash in a linear pattern that predominantly affected the left side of the body (Figure 1B). The lesions continually progressed, and a biopsy later revealed inflammatory linear verrucous epidermal nevus (Figure S1A). At ~1 year of age, she developed recurrent emesis and diarrhea. Repeat endoscopic biopsies demonstrated chronic, unspecified inflammation at various sites (most frequently colonic, but also gastric, duodenal, ileal, and esophageal regions). Eosinophilic infiltration of the colon was consistently noted (Figures 1C and S1B). Likewise, peripheral eosinophilia with a fluctuating pattern was frequently observed (Figure S1C). At 3 years of age, she developed rapid weight gain, edema, and proteinuria. Renal biopsy demonstrated membranous nephropathy (MN) (Figures 1D and S1D), which was refractory to treatment with corticosteroids, and later, cyclosporine and tacrolimus. According to her history, the nephrotic syndrome was ameliorated via an elemental diet, but this was not able to be consistently maintained. Of note, serology for known MN autoantibodies or other autoreactive antibodies (Figure S1E). Kidney transplantation was performed at age 11, but MN recurred within 1 year, followed by antibody-mediated rejection, requiring subsequent hemodialysis. Over this time, she also experienced asthma, food and environmental allergies, severely stunted growth with leg length discrepancy, and poor weight gain (for an extended clinical report, see STAR Methods).

Whole-Exome Sequencing Reveals a De Novo JAK1 Mutation

Given the overall healthy state of the parents and the early onset of disease in the patient, we hypothesized that either a recessive or de novo genetic mutation was the cause of the clinical syndrome (Figure 1E). We performed whole-exome sequencing on peripheral blood cells obtained from the patient and her parents. Subsequent variant analysis failed to produce any likely variants by a recessive model of inheritance (Table S1). Because of the asymmetric manifestations of disease, including limb length discrepancy and irregularly distributed dermatitis, we then considered the possibility of lower-read-frequency de novo mosaic mutations, which are typically excluded from common analysis pipelines. One candidate de novo variant, JAK1 c.2108G > T, which constituted 27% of the reads mapping to the region, was identified (Figures 1E and 1F). The presence of the c.2108G > T variant was confirmed by Sanger sequencing (Figure 1G), and this variant was absent from all of the publicly available genome sequences from healthy individuals. This mutation results in the substitution of serine to isoleucine at position 703 (S703I) in a highly conserved region (Figure S1F) and is predicted to be highly damaging (combined annotation-dependent depletion [CADD] score of 27.8). We then investigated the presence of c.2108G > T in non-hematopoietic tissues. We performed digital droplet PCR (ddPCR) with mutation-specific probes to estimate the fraction of cells carrying the mutation in different tissues. We identified the mutation at various frequencies in DNA from buccal swabs, granulocytes, peripheral blood mononuclear cells (PBMCs), and endoscopic biopsy samples fractionated into epithelia and associated immune cells (Figures 1H and S1G). These tissues represent all three germ layers, signifying that the mutation must have arisen in the first ~12 cell divisions between fertilization and gastrulation (Figure 1I) (Moore et al., 2015).

Allele Characterization Indicates that S703I Confers a GoF on JAK1

The S703I mutation localizes to the pseudokinase domain of JAK1, a putative regulatory domain (Figure 2A). Although S703I is located between the germline JAK1 mutations identified to date, these other mutations diverged in their downstream consequences (LoF and GoF), making functional predictions for S703I difficult (Del Bel et al., 2017; Eletto et al., 2016). To assess the possible pathogenicity of the mutation and its impact on JAK1 function, we transduced WT JAK1, S703I JAK1, and empty vector lentiviruses into U4C cells, a fibrosarcoma cell line previously selected to lack endogenous JAK1 (Pellegrini et al., 1989). Transduction with S703I JAK1, but not WT JAK1 or Luciferase, led to basal phosphorylation of STAT proteins and active target gene transcription in the absence of cytokine stimulation (Figures 2B–2E). S703I-transduced cells hyperresponded to IFN-α,
terms of both the proximal phosphorylation of STAT1 and STAT2 and the induction of IFN-stimulated genes (ISGs) (Figures 2B and 2C). Similarly, these cells hyperphosphorylated downstream STATs in response to IFN-γ or IL-6 (Figures 2D, 2E, and S2A).

For direct confirmation of the pathogenicity of the mutation in cells from the patient, we derived an EBV-immortalized B cell (B-EBV) line from the patient’s PBMCs. Given the mosaicism for JAK1 in the patient’s cells, individual lines were cloned from single cells to derive purely wild-type (WT) or S703I heterozygous mutant cells (Figure S2B). A comparison of STAT phosphorylation in patient WT and mutant B-EBV cells supported the GoF role of S703I JAK1, both at baseline and in response to cytokines (Figures 2F and 2G). The isogenic control derived from the same patient pinpointed S703I JAK1 as the probable pathogenic mutation in the patient’s genome. These results indicate that S703I is GoF for basal- and cytokine-induced STAT signaling.

S703I JAK1 Transactivates Partnering JAKs Independently of Its Own Kinase Activity

To dissect the mechanisms underlying upregulated STAT signaling, we assessed the impact of S703I on JAK auto-phosphorylation. Consistent with the increase in STAT phosphorylation, S703I JAK1 was itself hyperphosphorylated (Figure 3A). In addition, JAK2, TYK2, and JAK3 phosphorylation

Figure 1.  De Novo Mutation in JAK1 Identified in a Patient with Immunodysregulatory Syndrome

(A) Schematic representing clinical history of the patient, with gray bars representing the kinetics of each disease feature.

(B) Photograph of the dermatologic lesions on the arm.

(C) Histology of the cecal mucosa showing expansion of the lamina propria secondary to increased inflammatory cell infiltrate, with eosinophils in the lamina propria and crypt epithelium (arrows).

(D) Electron microscopy of a renal biopsy obtained during disease recurrence that demonstrates irregular glomerular basement membranes and subepithelial and intramembranous immune type dense deposits.

(E) Patient’s family pedigree.

(F) Whole-exome sequencing reads mapping to JAK1 locus c.2108, with variant nucleotides displayed in green.

(G) Representative chromatograms from 3 independent experiments of Sanger sequencing of peripheral blood DNA to confirm c.2108 G > T JAK1.

(H) Proportion of cells carrying the heterozygous JAK1 mutation, as estimated by digital droplet PCR with WT- and mutation-specific probes. DNA was obtained from bilateral cheek swabs, Ficoll-fractionated whole blood, and epithelial tissue isolated from a colonic biopsy (n = 1).

(I) Model for the development of the de novo mutation and its distribution into all 3 germ layers. See also Figure S1.
were upregulated (Figures 3B–3D and S2C), suggesting that the interacting JAK partners may also play a role in the GoF. We reasoned that partnering JAK activity could be overactivated by JAK1 S703I from two mechanisms: increased formation of the receptor complex or direct crosstalk between JAK proteins. We assessed whether JAK1 S703I allowed for more cytokine receptor at the surface, as the structural domains of JAK1 scaffold the receptor complex (Li et al., 2013). However, surface staining for the type I IFN receptor subunit (IFNAR2) demonstrated equivalent receptor expression in WT JAK1 and S703I JAK1 cells, indicating that JAK1 scaffolding of the receptor complex was unaffected by the patient’s mutation (Figures 3E and S2D).

Next, we hypothesized that the mutant JAK1 pseudokinase domain transactivated the kinase activity of JAK2 and TYK2. To investigate this mechanism, we mutated the ATP-binding site (K908A) of JAK1 to render it catalytically inactive. This well-characterized mutation retains the signaling capability of the receptor complex, making it possible to study signaling by the partnering JAKs in isolation (Eletto et al., 2016; Li et al., 2013). As expected, STAT phosphorylation was largely reduced in the absence of JAK1 activity (Figures 3F and 3G). However, following inactivation of the kinase domain of S703I JAK1 (S703I/K908A), an aberrant increase in STAT phosphorylation relative to kinase-inactivated JAK1 without the S703I mutation was observed upon cytokine stimulation (Figures 3F and 3G). This result indicates that S703I JAK1 transactivated JAK2 and TYK2, revealing that pseudokinase domains can regulate partnering JAKs in trans, in addition to traditionally understood cis-regulation (Babon et al., 2014). This mechanism was conserved with the other reported JAK1 GoF mutation (A634D, Del Bel e al 2017) (Figure 3H), but not to the JAK2 GoF mutation (V617F) that is common in hematologic malignancy (Figure S2E).

Finally, to clinically substantiate the importance of partnering JAK activity in JAK1 S703I (and other JAK-mediated diseases), we compared the potency of selective and non-selective JAK inhibitors, filgotinib and tofacitinib, respectively (Changelian et al., 2003; Rompaey et al., 2013). Unlike a pan-JAK inhibitor, a JAK1-specific inhibitor was unable to completely abrogate JAK1 S703I signaling owing to the continued signaling of partnering JAKs transactivated by the JAK1 pseudokinase (Figures 3I and S2F, schematic). These findings underscore the biochemical and clinical importance of trans-regulation and indicates that JAK selectivity need not mean clinical efficacy. This notion not only challenges the current dogma in drug development but it also proves that precision medicine must be used on a mutation-specific basis.
Ex Vivo Analysis Reveals a Cell-Intrinsic GoF

To more robustly investigate the consequences of S703I on immune cells, we performed mass cytometry (CyTOF) immunophenotyping on whole blood from the patient. Despite the central role of JAK1 in immune cell differentiation and proliferation, the patient’s immune cell distribution was largely within the normal range, barring a few exceptions (Figures 4A and 4B; Table S2). As detected clinically, eosinophils fluctuated to high counts (Figure S1C). B cells, however, trended toward lower frequencies than in healthy donors, but largely retained a normal distribution of naive, memory, and class-switched cells (Figures S3A–S3C). Natural killer (NK) cells exhibited an increase in CD56<sup>hi</sup> cells (>12-fold over healthy controls) (Figures 4B, S3E, and S3F), a subset understood to be less mature but highly proliferative and rapid in cytokine production as compared to the majority CD56<sup>lo</sup> subset with high cytotoxicity (Figures S3G and S3H) (Poli et al., 2009). Extensive phenotyping of the patient’s NK cells was performed concurrently with STAT1 GoF NK cells, which also demonstrated...
Figure 4. CyTOF Analysis Reveals Cytokine-, STAT-, and Cell-Type-Specific Gain of Function

(A) Representative (tSNE) plots generated from immunophenotyping CyTOF data of whole blood from 2 independent experiments.

(B) Manually gated CyTOF populations from the whole blood of 5 separate healthy controls and the patient on 2 separate occasions (2X) were quantified as the percentage of single cells and expressed as relative frequency (patient/controls). The gray bars indicate means with standard deviations of healthy donors, and colored bars indicate means with standard deviations of patient. Multiple t tests performed correcting for multiple comparisons using the Holm-Sidak method. ***p < 0.001.

(C) Relative MSI of phospho-STAT staining from intracellular phospho-CyTOF of whole blood from 4 healthy donors (n = 4) and the patient on 3 separate occasions (3X). The columns represent means and the error bars represent standard deviations.

(D) Ex vivo stimulation with IFN-α (100 IU/mL), IL-2 (50 ng/mL), and IL-4 (50 ng/mL) for 15 min. The color intensity indicates the log2 fold-change in MSI over the unstimulated healthy control for each cell type (n = 1).

See also Figures S3 and S4.
functional immaturity (Vargas-Hernandez et al., 2017). This analysis revealed that JAK1 GoF NK cells aligned phenotypically with prototypical immature “CD56bright” NK cells, unlike those from STAT1 GoF patients (Figure S3I). However, whether this phenotype results from the primary pathology or clinical intervention remains to be determined in this case.

We then performed phospho-CyTOF to analyze the phosphorylation of all STATs downstream of JAK1 in all of the major immune cells of whole blood. Given the ubiquitous expression and diverse signaling capabilities of JAK1, as well as the basal activity of S703I observed in vitro, we hypothesized that all STATs within all immune cells would be hyperphosphorylated at baseline. Heightened phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6 was observed, but not universally. Certain immune subsets, but not others, exhibited high basal phosphorylation of specific STATs (Figure 4C). For example, granulocytes from the patient displayed baseline STAT1 phosphorylation, but not STAT3 phosphorylation, whereas T cells displayed basal STAT3 phosphorylation but not STAT1 phosphorylation. STAT6 phosphorylation, however, was not upregulated in any immune subset, except B cells.

To evaluate the functional consequences of the observed basal phosphorylation, we assessed the expression of downstream genes from bulk PBMCs. We detected the elevated expression of downstream pSTAT target genes, including IFI27, IFIT1, MX1, and SIGLEC1 (Figure S4A). In addition, we tested non-hematopoietic tissues for baseline STAT phosphorylation by immunohistochemistry. In skin and gastrointestinal biopsies, but not renal biopsy, we detected highly phosphorylated STAT1 and STAT3 as compared to healthy samples (Figure S4B). This basal phosphorylation was observed in the apparent absence of any overt increase in circulating JAK-STAT cytokine concentrations (Figure S4C), suggesting that intrinsic S703I JAK1 activity drove this process, consistent with our in vitro results (Figures 2B–2F).

**Ex Vivo Cytokine Stimulation Generates Non-canonical Signaling Pathways in Patient Cells**

Whole blood was then stimulated ex vivo with a series of cytokines that engage JAK1 with various cytokine receptors, JAK partners, and downstream STAT targets. In response to IFN-α or IL-2, patient leukocytes hyperphosphorylated STAT1 and STAT3 or STAT5, respectively (Figures 4D and S4D and S4E, represented plots). By contrast, STAT6 phosphorylation in response to IL-4 was similar to that in healthy control cells, as seen in the baseline STAT6 data (Figure 4D). Likewise, IL-5 stimulation led to normal STAT5 phosphorylation (Figure S5F). These differential responses were also noted in patient B-EBV cells (Figures S5G–S5I). However, stimulation with either IL-2 or IL-4 induced the phosphorylation of STAT1 exclusively in patient cells, contrasting the canonical signaling cascade induced by these cytokines (Figure 4D). This non-canonical response suggests that STAT6 phosphorylation is restricted to patient cells, contrasting the canonical signaling cascade induced by these cytokines (Figure 4D). This non-canonical response suggests that S703I confers promiscuity onto JAK1, allowing it to transverse traditional signaling axes and establish non-canonical pathways. These results indicate that S703I JAK1 is a GoF mutation ex vivo, displaying both unexpected pathway promiscuity and specificity in the activation of STAT signaling.

**JAK1 Expression Is Restricted to a Single Allele across Immune Cells**

Given the mosaicism of a heterozygous mutation, we expected to observe some cells (i.e., homozygous WT JAK1) containing only WT JAK1 transcripts and others (i.e., heterozygous S703I JAK1) containing both WT and S703I JAK1 transcripts. However, we found that expression of the 2 alleles seemed almost mutually exclusive, as very few cells expressed both transcripts, as opposed to the ~50% that was expected given our genomic estimates (Figures 1D and 5F, left panel). By contrast, other genes...
Figure 5. Custom scRNA-Seq Maps JAK1 Allele Distribution, Transcriptomic Impact, and Expression Patterns

(A) tSNE plots and cell-type assignments from scRNA-seq of patient PBMCs, with an inDrops platform adapted to target the mutant JAK1 transcript. n = 4,763 cells.

(B) tSNE plots representing the subset of cells with sufficient JAK1 counts to be assigned putative JAK1 genotypes (based on transcript sequences). Cells in which any mutant transcript was detected above empirically determined thresholds (> 5 JAK1 transcripts) were assigned “S703I JAK1” (purple), while cells with only WT transcript detected were assigned “WT JAK1” (orange).

(C) Doughnut charts quantifying allele distribution in cells meeting genotyping criteria (cell count in the center), as in (B).

(D) Expression of the ISG IFI44L, a statistically significant differentially expressed gene in the comparison of WT JAK1 and S703I JAK1 genotyped cells.

(E) Gene set scores for IFN-α signaling in CD14+ monocytes.

(F) Number of unique transcripts detected per cell for the WT or S703I JAK1 allele (left) or a control variant GNLY rs12845 (right). The bubble size indicates the number of cells. The color coding indicates cells containing S703I JAK1 (purple), WT JAK1 without S703I JAK1 (orange), WT JAK1 with S703I JAK1 detected below threshold (yellow), or insufficient transcripts counts (gray).

(G) Transcript genotyping of JAK1 rs2230587 from healthy control PBMCs (n = 96) by single-cell qPCR with allele-specific probes. The histogram represents the relative frequency of cells expressing binned allele ratios as quantified by oligonucleotide standards.

(I) Single-cell qPCR transcript genotyping of control gene NACA (rs4902).

See also Figure S5.
from our dataset containing heterozygous variants exhibited the expected biallelic distribution (Figure 5F, right panel). This result suggests that JAK1 may be subject to monoallelic bias, a pattern that has only recently been recognized in a fraction of the autosomal transcriptome (Borel et al., 2015; Deng et al., 2014; Gimelbrant et al., 2007; Jeffries et al., 2012). We further tested this hypothesis of biased allele expression by sorting single cells from healthy donor PBMCs heterozygous for a synonymous SNP (rs2230587) of JAK1, adjacent to S703I. qPCR of isolated RNA with allele-specific probes revealed that relative expression of the two alleles was not normally distributed, but rather biased to one allele or the other (Figure 5G), unlike in a control gene (Figure 5H). A query of publicly available murine expression data revealed a similar allele restriction for JAK1, which, at least in mice, remains fixed over time (Savova et al., 2016). Whether the observed phenomenon in this patient represents transcriptional bursting or mitotically stable monoallelic expression and the potential impact on immune dysfunction remains to be fully determined. In either sense, these data perhaps indicate a departure from the classic genetic interpretation of heterozygosity and allow for a shift in understanding of the genetic penetrance of disease.

**Clinical and Biological Immune Dysfunction Can Be Rescued with Tofacitinib**

Having identified JAK1 hyperactivity as the putative driver of clinical disease in the patient, we then considered the use of JAK inhibitors for the clinical treatment of this patient. We compared the ability of the two US Food and Drug Administration (FDA)-approved pan-JAK inhibitors available at the time to reduce basal STAT phosphorylation in S703I-transduced U4C cells. Despite its lower relative potency against JAK1, tofacitinib inhibited STAT phosphorylation in a comparable dose response to ruxolitinib (Figure 6A), again reflecting the independence of JAK1 catalysis and STAT hyperphosphorylation. Similar results were obtained with patient-derived B-EBV cells, which, unlike the transduced fibrosarcoma U4C cells, are hematopoietic in origin and therefore express JAK3 (Figure 6B). Next, we treated patient blood ex vivo with the two compounds at equimolar doses that mimic physiological dosing (Chen et al., 2014; Krishnaswami et al., 2014; Lamba et al., 2016), and we further assessed the inhibition of IFN-α stimulation. Analysis of phospho-STAT inhibition across whole-blood immune subsets by phospho-CyTOF revealed that tofacitinib attenuated the response more potently than ruxolitinib in nearly all cell types (Figure 6C).

Following these extensive functional studies, we treated the patient with low-dose tofacitinib (5 mg daily). Within 8 weeks, circulating inflammatory markers (erythrocyte sedimentation rate [ESR] and C-reactive protein) normalized (Figures 6D and 6E). Near-complete improvement in dermatitis followed, both grossly and histologically (Figure 6F). By 6 months, the patient reported complete resolution of gastrointestinal symptoms (decrease in modified Pediatric Ulcerative Colitis Activity Index [PUCAI] from 35–50 initially to 0). Biopsy of colonic tissue revealed the restoration of crypt architecture and the complete resolution of eosinophilic infiltrates (Figure 6G). The patient remained stable for 2 years after initiation of therapy until, unfortunately, the patient succumbed to acute respiratory failure due to coronavirus disease 2019 (COVID-19).

Finally, we confirmed the pharmacological rescue of JAK hyperactivity in the patient’s cells after tofacitinib treatment. RNA isolated from PBMCs revealed that the expression of ISGs, which was elevated before treatment, progressively declined to normal levels (Figure 6H). CyTOF analysis was then performed to confirm the decrease in basal STAT phosphorylation (Figure 9E). Reductions were observed across cell types in STAT3, STAT4, STAT5, and STAT6 phosphorylation, whereas STAT1 phosphorylation was reduced in some, but not all cell types (Figure 6I). Overall, these results validate S703I JAK1 as the etiology in vivo of the widespread immune dysregulation (summarized in Table S4), and they illustrate the power of precision medicine both as a treatment approach for patients with rare diseases and as a means of discovering fundamental physiological mechanisms.

**DISCUSSION**

Undiagnosed disease programs have shown promise for the detection of potential causative variants of disease (Lee et al., 2014; Splinter et al., 2018; Yang et al., 2014). This report demonstrates the value of the in-depth study of select patients identified in these programs. Most immediately, the findings described herein directed the successful treatment of a complex immunodysregulatory disease in a personalized molecular fashion. More broadly, this case implicates JAK1 dysfunction in common forms of multifactorial diseases, including dermatitis, enteritis, colitis, and eosinophilic disorders. These features align with the other reported JAK1 GoF mutation recently published (Del Bel et al., 2017), and together provide strong justification for the expanding use of JAK inhibitors in these disorders (O’Shea and Gadina, 2019; O’Shea et al., 2015). However, to date, MN has not been recognized to involve JAK-STAT dysregulation. Identifying more patients with JAK1 GoF mutations will be critical to determine whether the link between JAK1 GoF and MN is causal. If true, then MN, the most common cause of nephrotic syndrome, may be amenable to early treatment with JAK inhibitors. In fact, baricitinib, a JAK1 and JAK2 inhibitor, demonstrated recent success in a clinical trial for diabetic nephropathy, a distinct but related nephrotic syndrome (Tuttle et al., 2018).

The absence of MN in the other reported JAK1 GoF mutation (A634D) may represent important distinctions in the behavior of different mutated forms of JAK1, rather than variable penetrance of the same genetic etiology. Disruptions of the pseudokinase domain may have vastly divergent functional consequences, which can already be gleaned by comparing the JAK1 mutations identified to date: P733L and P832S (LoF) versus A634D and S703I (GoF). Moreover, our findings indicate that GoF may not necessarily lead to the universal activation of downstream pathways, as S703I caused the hyperactivation of some pathways, but not others. Furthermore, disruption of the pseudokinase domain by S703I enabled cells to respond promiscuously via non-canonical signaling pathways. These findings suggest that the pseudokinase domain is not a simple “on/off” switch. This regulatory complexity may stem from the ability of the JAK1 pseudokinase to modulate the activity of JAK2 and TYK2, as demonstrated here. Consequently, careful study of each mutation is warranted, each yielding valuable information on fundamental JAK1 function. The complex regulation becomes...
especially important, given the high incidence of oncogenic JAK mutations, as well as the expanding therapeutic use of JAK inhibitors. Regarding the latter, the evidence presented here and elsewhere (Eletto et al., 2016; Haan et al., 2011; Li et al., 2013) of the highly cooperative action of JAKs challenges the strategic wisdom of increasing the selectivity of JAK inhibitors.

Following transactivation of partnering JAKs, S703I JAK1 constitutively upregulated STAT1, STAT2, STAT3, STAT4, and STAT5 phosphorylation and their downstream target genes, all in the apparent absence of circulating cytokine. These STATs mediate signaling for >25 cytokine pathways, and thus the clinical disease that results is likely driven by a complex mixture of...
these pathways. Most predominantly, the pathophysiology in this patient appeared to be a combined (1) autoinflammatory and (2) atopic disease process. The presence of severe, early-onset unspecified inflammation throughout the gastrointestinal tract and skin with drastically high acute phase reactants is evocative of primary autoinflammatory disorders. In this regard, the JAK1 GoF here represents a partial phenocopy of other systemic autoinflammatory diseases that overactivate the IFN and IL-6 axes. These include STAT3 GoF mutations (Fabre et al., 2019) and the type I interferonopathies, disorders of overactive STAT1 and STAT2 activity (Rodero and Crow, 2016)—pathways that were highly upregulated by JAK1 S703I. Other disease features suggested an atopic syndrome. Eosinophils were elevated in peripheral circulation and infiltrating into the gastrointestinal lamina propria and crypt epithelium. Asthma, food and environmental allergies, and a skin disease similar to atopic dermatitis were severe and early in onset. Lastly, her renal disease (membranous nephropathy) could only be ameliorated by an elemental diet of strictly liquid nutrients, suggesting a dietary hypersensitivity trigger. Considering the high basal phosphorylation of STAT5 in this patient, these features seem to phenocopy GoF STAT5b (Ma et al., 2017) mutations, which result in early-onset eosinophilia, urticaria, dermatitis, and diarrhea. While it is possible the pathological inflammatory stage of COVID-19 pathogenesis was exaggerated in this patient, other risk factors must be considered, including the comorbidities (e.g., asthma, renal failure) and the combined immunosuppression (i.e., tofacitinib, tacrolimus, and oral glucocorticoids) in this patient. In particular, there is an urgent need to understand how JAK inhibition modulates both the protective and pathological inflammatory immune responses to SARS-CoV-2, as JAK inhibitors are in clinical trials for COVID-19.

Finally, this study advances our understanding of mosaicism—at both a genomic and transcriptomic level—in personalized medicine. Genomically, this JAK1 mutation was uncovered, mapped across tissues to trace its embryological origin, and directly linked to phenotypic consequences by deriving JAK1-WT and JAK1-S703I cell lines from the patient. These findings underscore how asymmetric clinical manifestations like those observed here (leg-length discrepancy and dermatitis along lines of embryological migration) should prompt suspicions of mosaicism and guide genetic analysis with carefully chosen techniques. In particular, the analyses described here have only recently become possible with technological advances in single-cell assays. Here, by adapting the inDrops single cell RNA-seq platform, we implement an approach for the detection and analysis of a specific mutation within a transcript region not readily accessible by standard methods. The resulting single-cell resolution data allowed us to determine mutant allele frequency in different cell populations, identify mutation-associated gene expression patterns, and, unexpectedly, to observe allelic bias in the transcription of JAK1. This bias is consistent with recent evidence of widespread transcriptional bursting and monoallelic expression of autosomal genes (Borel et al., 2015; Gimelbrant et al., 2007; Jeffries et al., 2012; Reinius and Sandberg, 2015). This report demonstrates monoallelic expression of a mutated gene. Biased allelic expression, in conjunction with mosaicism, may prove an important point of focus for future genetic studies of variable penetrance, affected carriers, and undiagnosed disease.

In conclusion, intense basic mechanistic investigations of a single mutation identified pan-JAK inhibition, as opposed to highly selective JAK1 inhibition, as the optimal therapy for personalized medicine, leading to biological and clinical rescue. This approach constitutes a workflow for alike monogenic syndromes.

**STAR★METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at [https://doi.org/10.1016/j.immuni.2020.07.006](https://doi.org/10.1016/j.immuni.2020.07.006).

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-JAK1 Clone B-3 | Santa Cruz Biotechnology | Cat No. sc-376996; AB_2687564 |
| Rabbit anti-JAK2 Clone D2E12 | Cell Signaling Technology | Cat No. 3230T; AB_10691469 |
| Rabbit anti-phospho-JAK2 Tyr1007/1008 | Cell Signaling Technology | Cat No 3771S; RRID:AB_330403 |
| Rabbit anti-JAK3 Clone D1H3 | Cell Signaling Technology | Cat No. 8827S; RRID:AB_10999548 |
| Rabbit anti-TYK2 Clone D415T | Cell Signaling Technology | Cat No. 14193S; RRID:AB_2798419 |
| Rabbit anti-phospho-TYK2 Tyr1054/1055 | Cell Signaling Technology | Cat No. 9321; RRID:AB_2303972 |
| Mouse anti-phospho-tyrosine Clone 4G10 | Millipore Sigma | Cat No. 05-321X |
| Mouse anti-STAT1 Clone C-111 | Santa Cruz Biotechnology | Cat No. sc417; RRID:AB_675902 |
| Rabbit anti-STAT2 | Millipore Sigma | Cat No. 06502; RRID:AB_31014 |
| Rabbit anti-phospho-Tyr 701-STAT1 Clone 58D6 | Cell Signaling Technology | Cat No. 9167; RRID:AB_561284 |
| Rabbit anti-phospho-Tyr-689-STAT2 Clone D3P2P | Cell Signaling Technology | Cat No. 88410; RRID:AB_2800123 |
| Rabbit anti-phospho-STAT6 Tyr694 Clone C11C5 | Cell Signaling Technology | Cat No. 9359S; RRID:AB_823649 |
| Rabbit anti-phospho-STAT5 Tyr694 Clone C11C5 | Cell Signaling Technology | Cat No. 9361T; RRID:AB_331595 |
| Rabbit anti-phospho-tyrosine Clone 4G10 | Millipore Sigma | Cat No. 05-321X |
| Mouse anti-GAPDH Clone D16H11 | Cell Signaling Technology | Cat No. 5174; RRID:AB_10622025 |
| Go anti-mouse IgG HRP-conjugated | Southern Biotech | Cat No. 101005; RRID:AB_2687483 |
| Go anti-rabbit IgG HRP-conjugated | Southern Biotech | Cat No. 403005; RRID:AB_2687483 |
| Rabbit anti-IFNAR2 MMHAR-2 | PBL | Cat No. 21385-1; RRID:AB_387828 |
| Discovery OmniMap anti-rabbit HRP (RUO) | Roche | Cat No. 760-4311; RRID:AB_2811043 |
| anti-IgD 141Pr-conjugated Clone IA6-02 | Biolegend | Cat No. 348202; RRID:AB_10550095 |
| anti-CD19 142Nd-conjugated Clone HIB19 | Biolegend | Cat No. 302202; RRID:AB_266181 |
| anti-CD45RA 143Nd-conjugated Clone H100 | Biolegend | Cat No. 304102; RRID:AB_314406 |
| anti-CD44 144Nd-conjugated Clone M80 | Biolegend | Cat No. 344102; RRID:AB_2661788 |
| anti-CD4 145Nd-conjugated Clone RPA-T4 | Biolegend | Cat No. 300502; RRID:AB_314070 |
| anti-CD8 146Nd-conjugated Clone RPA-T8 | Biolegend | Cat No. 301002; RRID:AB_2661818 |
| anti-CD20 147Sm-conjugated Clone 2H7 | Biolegend | Cat No. 302302; RRID:AB_314250 |
| anti-CD16 148Nd-conjugated Clone 3G8 | Biolegend | Cat No. 302014; RRID:AB_314214 |
| anti-CD127 149Sm-conjugated Clone A019D5 | Fluidigm | Cat No. 3149011B; RRID:AB_2661792 |
| anti-CD1c 150Nd-conjugated Clone L161 | Biolegend | Cat No. 331502; RRID:AB_2661820 |
| anti-CD123 151Eu-conjugated Clone 6H6 | Biolegend | Cat No. 306002; RRID:AB_2661822 |
| anti-CD66b 152Sm-conjugated Clone G10F5 | Biolegend | Cat No. 305102; RRID:AB_2661823 |
| anti-PD1 153Eu-conjugated Clone EH12.2H7 | Biolegend | Cat No. 329926; RRID:AB_1147365 |
| anti-CD86 154Sm-conjugated Clone IT2.2 | Biolegend | Cat No. 305410; RRID:AB_314530 |
| anti-CD27 155Gd-conjugated Clone O323 | Biolegend | Cat No. 302802; RRID:AB_2661825 |
| anti-PDL1 156Gd-conjugated Clone 29E.2A3 | Biolegend | Cat No. 329770; RRID:AB_2275581 |
| anti-CD33 158Gd-conjugated Clone WM53 | Biolegend | Cat No. 303402; RRID:AB_314346 |
| anti-CD24 159Th-conjugated Clone ML5 | Biolegend | Cat No. 311102; RRID:AB_314851 |
| anti-CD14 160Gd-conjugated Clone M5E2 | Biolegend | Cat No. 301810; RRID:AB_31419 |
| anti-CD56 161Dy-conjugated Clone B159 | BD Biosciences | Cat No. 555513; RRID:AB_395903 |
| anti-CD169 162Dy-conjugated Clone 7-239 | Biolegend | Cat No. 346002; RRID:AB_2198031 |
| anti-CCR5 163Dy-conjugated Clone REA103 | Miltenyi | Cat No. 130-122-325; RRID:AB_2801905 |
| anti-CD69 164Dy-conjugated Clone FN50 | Biolegend | Cat No. 310902; RRID:AB_314837 |
| anti-CCR6 165Ho-conjugated Clone G034E3 | Biolegend | Cat No. 353402; RRID:AB_10918625 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| anti-CD25 166Er-conjugated Clone M-A251 | Biolegend | Cat No.356102; RRID:AB_2661833 |
| anti-CCR7 167Er-conjugated Clone G043H7 | Biolegend | Cat No.353256; RRID:AB_2814291 |
| anti-CD3 168Er-conjugated Clone UCHT1 | Biolegend | Cat No.300402; RRID:AB_2661835 |
| anti-CX3CR1 169Tm-conjugated Clone 2A9-1 | Biolegend | Cat No.341602; RRID:AB_1595422 |
| anti-CD38 170Er-conjugated Clone HB-7 | Biolegend | Cat No.356602; RRID:AB_2661836 |
| anti-CD161 171Yb-conjugated Clone HP-3G10 | Biolegend | Cat No.339902; RRID:AB_2661837 |
| anti-CD209 172Yb-conjugated Clone 9E9A8 | Biolegend | Cat No.330102; RRID:AB_1134253 |
| anti-CXCR3 173Yb-conjugated Clone REA232 | Miltenyi | Cat No.130-108-022; RRID:AB_2655743 |
| anti-HLADR 174Yb-conjugated Clone L243 | Biolegend | Cat No.307602; RRID:AB_314680 |
| anti-Axl 175Lu-conjugated Clone 108724 | R&D Systems | Cat No.MAB154; RRID:AB_2062558 |
| anti-CCR4 176Yb-conjugated Clone 205410 | R&D Systems | Cat No.MAB1567; RRID:AB_2074395 |
| anti-pSTAT5 147 Sm-conjugated Clone 47 | Fluidigm | Cat No.3147012A; RRID:AB_2827887 |
| anti-pSTAT6 149 Sm-conjugated Clone 18 | Fluidigm | Cat No.3149004A |
| anti-pSTAT1 153 Eu-conjugated Clone 4a | Fluidigm | Cat No.3153005A; RRID:AB_2744689 |
| anti-pp38 156 Gd-conjugated Clone D3F9 | Fluidigm | Cat No.3156002A; RRID:AB_2661826 |
| anti-pSTAT3 158 Gd-conjugated Clone 4 | Fluidigm | Cat No.3158005A; RRID:AB_2811100 |
| anti-pMAPKAP2 159 Tb-conjugated Clone 27B7 | Fluidigm | Cat No.319010A; RRID:AB_2661828 |
| anti-STAT3 165 Ho-conjugated Clone 124H6 | Fluidigm | Cat No.3173003A |
| anti-STAT1 169 Tm-conjugated Clone 104B40 | Biolegend | Cat No.661002; RRID:AB_2563664 |
| anti-pERK 171 Yb-conjugated Clone D13.14.4E | Fluidigm | Cat No.3171010A; RRID:AB_2811250 |
| anti-pS6 175 Lu-conjugated Clone N7-548 | Fluidigm | Cat No.3175009A; RRID:AB_2811251 |
| Mouse anti-NKp46 Pacific Blue Clone 900 | BioLegend | Cat No.331912; RRID:AB_2149280 |
| Mouse anti-CD56 Brilliant Violet 605 Clone HCD56 | BioLegend | Cat No.318334; RRID:AB_2561912 |
| Mouse anti-CD16 Brilliant Violet 650 Clone 3G8 | BioLegend | Cat No.302041; RRID:AB_11125578 |
| Mouse anti-CD3 Brilliant Violet 711 Clone OKT3 | BioLegend | Cat No.317328; RRID:AB_2562907 |
| Mouse anti-CD8-alpha Brilliant Violet 785 Clone RPA-T8 | BioLegend | Cat No.301046; RRID:AB_2563264 |
| Mouse anti-CD107a FITC Clone eBioH4A3 | eBioscience | Cat No.53-1079-42; RRID:AB_2016657 |
| Mouse anti-NKp44 PE Clone Z231 | Beckman Coulter | Cat No. IM3710; RRID:AB_2857937 |
| Mouse anti-NK2G2D PE-Cy7 Clone 1D11 | BioLegend | Cat No.320811; RRID:AB_2132375 |
| Mouse anti-CD69 PE-CF594 Clone FN50 | BD Biosciences | Cat No.562617; RRID:AB_2737680 |
| Mouse anti-NKp30 APC Clone P30-15 | BioLegend | Cat No.325209; RRID:AB_2149450 |
| Mouse anti-CD16 APC Cy7 Clone B73.1 | BD Biosciences | Cat No.561306; RRID:AB_10643005 |
| Mouse anti-CD25 APC-Alexa Fluor 700 Clone B1.49.9 | Beckman Coulter | Cat No. A86356; |
| Rat anti-CD2 Pacific Blue Clone 39C1.5 | Beckman Coulter | Cat No. B09685; RRID:AB_2847880 |
| Mouse anti-CD244 FITC Clone 25235 | BD Biosciences | Cat No.550815; RRID:AB_393900 |
| Mouse anti-CD11c PerCP Cy5.5 Clone BU15 | Beckman Coulter | Cat No. B19719; |
| Mouse anti-CD28 PE Clone L293 | BD Biosciences | Cat No.348047; RRID:AB_400368 |
| Mouse anti-CD11a PE-Cy7 Clone H111 | BD Biosciences | Cat No.561387; RRID:AB_10611572 |
| Mouse anti-CD54 PE-Cy5 Clone HA58 | BD Biosciences | Cat No.555512; RRID:AB_395902 |
| Mouse anti-CD11b PE-CF594 Clone ICRF44 | BD Biosciences | Cat No.562399; RRID:AB_2737613 |
| Mouse anti-CD18 APC Clone 6.7 | BD Biosciences | Cat No.551060; RRID:AB_398485 |
| Mouse anti-CD158e Brilliant Violet 421 Clone DX9 | BioLegend | Cat No.312714; RRID:AB_312714 |
| Mouse anti-CD158b FITC Clone DX27 | BioLegend | Cat No.312604; RRID:AB_2296486 |
| Mouse anti-CD94 PerCP Cy5.5 Clone HP-3D9 | BD Biosciences | Cat No.562361; RRID:AB_11152081 |
| Mouse anti-NKG2C PE Clone 134691 | R&D Systems | Cat No. FAB138P; RRID:AB_2132983 |
| Mouse anti-CD158a/h/g PE-Cy7 Clone HP-MA4 | Affymetrix eBioscience | Cat No. 25-1589-42; RRID:AB_10854424 |
| Mouse anti-KIR2DS4 APC Clone 179315 | R&D Systems | Cat No. FAB8147A; RRID:AB_2130821 |
| Recombinant anti-KLRG1 APC-Vio770 Clone REA226 | Miltenyi Biotec | Cat No. 130103642; RRID:AB_2652580 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse anti-NKG2A Alexa Fluor 700 Clone 131411 | R&D Systems | Cat No. FAB1059N; RRID:AB_10972129 |
| Mouse anti-CD57 Pacific Blue Clone NC1 | Beckman Coulter | Cat No. A74779 ; RRID:AB_131600 |
| Mouse anti-CD62L Brilliant Violet 650 Clone DREG-56 | BioLegend | Cat No. 304831; RRID:AB_2561461 |
| Mouse anti-CD127 Brilliant Violet 785 Clone A019D5 | BioLegend | Cat No. 351330; RRID:AB_2563605 |
| Mouse anti-IL-15R-alpha Alexa Fluor 488 Clone 151303 | R&D Systems | Cat No. FAB1471G; RRID:AB_10891330 |
| Mouse anti-CD117 PE-Cy7 Clone 104D2D1 | Beckman Coulter | Cat No. IM3698; RRID:AB_131184 |
| Mouse anti-CD16 PE-CF594 Clone 3G8 | BD Biosciences | Cat No. 562293; RRID:AB_11151916 |
| Mouse anti-CD94 APC Clone DX22 | BioLegend | Cat No. 305508; RRID:AB_2133129 |
| Mouse anti-Perforin Brilliant Violet 421 Clone B-D48 | BioLegend | Cat No. 353307; RRID:AB_11149688 |
| Mouse anti-IFN-gamma Alexa Fluor 700 Clone 4S.B3 | BioLegend | Cat No. 502520; RRID:AB_528921 |
| Mouse anti-Eomes eFluor 660 Clone WD1928 | eBioscience | Cat No. 50-4877-42; RRID:AB_2574229 |
| Mouse anti-CD34 Alexa Fluor 700 Clone 4S.B3 | BioLegend | Cat No. 343526; RRID:AB_2561495 |
| Mouse anti-Perforin APC Cy7 Clone dG9 | BioLegend | Cat No. 308128; RRID:AB_2572051 |
| Mouse anti-Helios Brilliant Violet 421 Clone 22F6 | BioLegend | Cat No. 137233; RRID:AB_2565798 |
| Mouse anti-T-bet FITC Clone 4B10 | BioLegend | Cat No. 644812; RRID:AB_2200540 |
| Mouse anti-IRF8 PercP- eFluor 710 Clone V3GYWCH | eBioscience | Cat No. 46-9852-80; RRID:AB_2573903 |
| Mouse anti-GATA3 PE Cy7 Clone L50-823 | BD Biosciences | Cat No. 560405; RRID:AB_1645344 |
| Mouse anti-NKp44 PE Clone P44-8 | BioLegend | Cat No. 325107; RRID:AB_756099 |
| Goat anti-mouse IgG Alexa Fluor 647 | Thermo Fisher | Cat No. A-21235; RRID:AB_2535804 |

**Bacterial and Virus Strains**

- **DH5-Alpha Competent E. Coli**: Molecular Cloning Laboratories Cat No. DA-196

**Biological Samples**

- **Human whole blood samples**: Various institutions N/A

**Chemicals, Peptides, and Recombinant Proteins**

- **Intron-A Recombinant Interferon Alpha-2b**: Merck Pharmaceuticals Cat No. NDC0085057102
- **Recombinant Human IL-2**: BioLegend Cat No. 589102
- **Recombinant Human IL-4**: BioLegend Cat No. 574002
- **Recombinant Human IL-5**: BioLegend Cat No. 560701
- **Recombinant Human IL-6**: BioLegend Cat No. 570802
- **Recombinant Human IL-13**: BioLegend Cat No. 571102
- **Recombinant Human IL-21**: BioLegend Cat No. 571202
- **Recombinant Human Interferon Gamma**: BioLegend Cat No. 570206
- **phorbol 12-myristate 13-acetate**: Sigma-Aldrich Cat No. P1585
- **Ionomycin**: Sigma-Aldrich Cat No. I0634
- **Brefeldin A**: Sigma-Aldrich Cat No. B7651
- **Ruxolitinib**: Selleckchem Cat No. S1378
- **Tofacitinib**: Selleckchem Cat No. S5001
- **Filgotinib**: Selleckchem Cat No. S7605
- **Proteomic Stabilizer Prot1**: SMART TUBE Inc Cat No. 501351691
- **Heparin**: Sigma Cat No. 201060
- **Osmium tetroxide (99.9%)**: ACROS organics Cat No. 191180010
- **Cell-ID 20-plex Pd Barcoding Kit**: Fluidigm Cat No. 201060
- **Human TruStain FcX (Fc Receptor Blocking Solution)**: BioLegend Cat No. 422301
- **X8 MaxPar conjugation kits**: Fluidigm Cat No. 201300
- **125nM Ir Intercalator**: Fluidigm Cat No. 201192A
- **OsO4**: ACROS Organics Cat No. AC319010050
- **Maxpar Cell Acquisition Buffer**: Fluidigm Cat No. 201241
- **Cytofix/Cytoperm Fixation/Permeabilization Solution**: BD Cat No. 554714
- **FoxP3 Transcription Factor Staining Buffer**: Tonbo Cat No. TNB0607

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Live/Dead Aqua      | Thermo Fisher | Cat No. L34957 |
| Discovery Ultra antibody block | Roche | Cat No. 760-4204 |
| Pierce ECL Western Blotting Substrate | Thermo Fisher Scientific | Cat No. 32106 |
| Discovery Ultra Antibody Block | Roche | Cat No. 760-4204 |
| Protease/Phosphatase Inhibitor Cocktail | Cell Signaling Technologies | Cat No. 5872 |
| Nupage Western Blot Sample Buffer | Thermo Fisher Scientific | Cat No. NP0007 |
| Dynabeads Protein G | Thermo Fisher Scientific | Cat No. 10007D |
| Histopaque 1077     | Millipore Sigma | Cat No. 10771-500 |
| Cyclosporin         | Sigma Aldrich | Cat No. C3662 |
| OptiPrep Density Gradient Medium | Sigma Aldrich | Cat No. D1556-250mL |
| Novec 7500 Engineered Fluid | 3M | Cat No. Novec 7500 |
| 1H,1H, 2H, 2H – Perfluorooctanol, 97% | Alfa Aesar, Thermo Fisher Scientific | CAS No. 647-42-7 |
| 50 g of 10 weight % 008-Flurorosurfactant in HFE7500 Ran Biotechnologies | 008-FlurorSurfactant-10wtH-50G |
| Exonuclease I (E. Coli) | New England Biolabs | M0293L |
| Fast Digest HinfI | Thermo Fisher Scientific | FD0804 |
| AMPure XP | Beckman Coulter | A63881 |
| RNAClean XP | Beckman Coulter | A66514 |
| RNaseH | New England Biolabs | M0297L |

Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| QuikChange II XL site-directed mutagenesis kit | Agilent Technologies | Cat No. 200522 |
| Gateway LR Clonase II Enzyme Mix | Thermo Fisher Scientific | Cat No. 11791100 |
| Gateway BP Clonase Enzyme Mix | Thermo Fisher Scientific | Cat No. 11789020 |
| QIAamp DNA Mini Kit | QIAGEN | Cat No. 51304 |
| RNeasy RNA Isolation Kit | QIAGEN | Cat No. 74106 |
| Applied Biosystems High-Capacity cDNA Reverse | Thermo Fisher Scientific | Cat No. 4368814 |
| Transcription Kit |
| TaqMan Universal Master Mix II with UNG | Thermo Fisher Scientific | Cat No. 4440093 |
| Human Magnetic Luminex Assay Custom | R&D | Cat No. LXSAMH |
| Bio-Plex Pro Human Inflammation Panel | BioRad | Cat No. 171al001m |
| Cell-ID 20-Plex Pd Barcoding Kit | Fluidigm | Cat No. 201060 |
| Chromium single cell Chip Kit V2 | 10X Genomics | Cat No. 120236 |
| Chromium single cell 3’ Library and Gel Bead Kit | 10X Genomics | Cat No. 120237 |
| Discovery ChromoMap DAB Kit | Roche | Cat No. 760-159 |
| MycoAlert PLUS Mycoplasma Detection Kit | Lonza | Cat No. LT07-703 |
| Lipofectamine 3000 Transfection Reagent | Thermo Fisher | Cat No. L3000001 |
| Invitrogen Taq polymerase | Thermo Fisher | Cat No. 10342020 |
| Digital droplet PCR Supermix | Biorad | Cat No. 1863026 |
| IgG Autoantibody Array | RayBiotech | Cat No. PAH-AIDG-G1-16 |
| Single Cell Lysis Buffer | Ambion | Cat No. 4458235 |
| SuperScript VILO RT kit | Thermo Fisher | Cat No. 11754050 |
| SuperScript™ III Reverse Transcriptase | Thermo Fisher Scientific | Cat No. 1808093 |
| HiScribe T7 High Yield RNA Synthesis Kit | New England Biolabs | Cat No. E2040S |
| Kapa HiFi HotStart ReadyMix PC | Roche | KR0370 |
| NextSeq 500/550 High Output Kit v2.5 (75 cycles) | Illumina | 20224906 |

Deposited Data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| scRNA-seq Data and Analysis | This paper | https://github.com/jorgcalis/JAK1-alleles-pipeline |

(Continued on next page)
### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HEK293T             | ATCC   | CRL-3216   |
| OP9                 | ATCC   | CRL-2749   |
| U4C (JAK1−/−)       | Sandra Pellegrini | N/A |
| γ2A (JAK2−/−)       | Sandra Pellegrini | N/A |
| EBV-immortalized B cells | Icahn School of Medicine at Mount Sinai | N/A |

### Oligonucleotides

| Oligonucleotide | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| MX1 mRNA TaqMan FAM | Thermo Fisher | Cat No. 4331182 |
| RSAD2 mRNA TaqMan FAM | Thermo Fisher | Cat No. 4351370 |
| scRNAseq polyT oligonucleotide template | Integrated DNA Technologies | N/A |
| scRNAseq JAK1 oligonucleotide template | Integrated DNA Technologies | N/A |
| scRNAseq JAK1 blocking oligonucleotide | Integrated DNA Technologies | N/A |
| scRNAseq second reverse transcription JAK1 primer | Integrated DNA Technologies | N/A |
| JAK1 single cell qPCR amplification (F: GTCGCGACCTTCATGCA, R: GCTGTTGTGGCCATTCAAGC) | Thermo Fisher | N/A |
| JAK1 single cell genotyping probes (AAGGACATC[G/A]CTTTC) | Thermo Fisher | N/A |

### Recombinant DNA

| Reagent | SOURCE | IDENTIFIER |
|---------|--------|------------|
| pTRP IRES RFP PuroR | This paper | N/A |
| pDONR223 JAK1 | AddGene | Cat No. 23932 |
| pDONR223 JAK2 | AddGene | Cat No. 23915 |
| pCAGGS-VSV-G | This paper | N/A |
| pCMV-Gag/Pol | This paper | N/A |

### Software and Algorithms

| Software | SOURCE | IDENTIFIER |
|----------|--------|------------|
| Cytobank | Beckman Coulter | https://www.cytobank.org |
| FlowJo | Becton Dickinson Company | https://www.flowjo.com |
| R for Statistical Computing | The R Foundation | https://rstudio.com |
| CellRanger | 10X Genomics | https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger |
| Python v2.7 | Python | https://www.python.org/ |
| Seurat v2.3.4 R toolkit | Satija Lab, New York Genome Center | https://satijalab.org/seurat/ |
| EdgeR v3.12.1 | Bioconductor | http://bioinf.wehi.edu.au/edgeR/ |
| inDrops.py v0.3 | GitHub | https://github.com/indrops/indrops |
| Genome Analysis Toolkit (GATK) | Broad Institute | https://gatk.broadinstitute.org/hc/en-us |
| Ingenuity Variant Analysis | QIAGEN | https://variants.ingenius.com |
| GraphPad Prism 7 | GraphPad Software | https://www.graphpad.com/scientific-software/prism/ |
| QX100 software | BioRad | N/A |
| Genepix Pro 7.0 software | Molecular Devices | N/A |
RESOURCES AVAILABILITY

Lead Contact
Further information and requests for reagents may be directed to the Lead Contact, Dusan Bogunovic (dusan.bogunovic@mssm.edu).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
The raw data for experiments performed, including the PCR, multiplex ELISA, mass cytometry, flow cytometry, scRNA-seq data, are available upon request from the lead contact. For privacy concerns of the study participants, raw scRNA-Seq data are not available, and the complete data files from whole exome sequencing will be restricted to the variants in Table S1. scRNA-Seq gene x cell matrices, python scripts and associated Seurat code (R-based) used for the tailored analysis of JAK1-specific scRNA-Seq are available at https://github.com/jorgcalis/JAK1-alleles-pipeline.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients
This study reports an 18-year-old female who presented during early childhood with persistent, recurrent cutaneous and gastrointestinal inflammatory disease with eosinophilic infiltration and peripheral eosinophilia. Notable physical features include a leg length discrepancy, short stature and low body weight. In parallel, she developed refractory membranous nephropathy leading to end stage renal disease. A kidney transplant was complicated by disease recurrence in the graft, progressing over several years, as well as acute rejection ultimately rendering the patient dialysis-dependent. Specifically, her past medical history by organ involvement is further detailed, as follows:

Renal: At 3 years of age, she developed rapid weight gain, edema and proteinuria. Renal biopsy at age 7 demonstrated membranous nephropathy, which was refractory to treatment with corticosteroids, and later cyclosporine and tacrolimus. By history the nephrotic syndrome was ameliorated by use of an elemental diet, but this was not able to be consistently maintained. Serological testing for anti-PLA-2R receptor, anti-thrombospondin and anti-bovine serum albumin was all negative. A gradual decline in renal function was observed and at age 11 a living-donor kidney transplantation was performed. One year later, nephrotic range proteinuria recurred, and biopsy confirmed relapsing membranous nephropathy. The graft function further declined, and an episode of acute antibody-mediated rejection resulted in transplant failure at age 16. She has required long-term hemodialysis since that time, delivered via an AV fistula, and is currently being evaluated for a second transplant.

Dermatologic: At birth, a pustular rash involving face and extremities was noted. The rash persisted and worsened after discharge. Skin biopsy at 3 months suggested Inflammatory Linear Verrucous Nevus. Skin involvement spread and worsened in intensity with age, manifesting as a diffuse, erythematous rash involving the face, trunk and extremities, with prominence on the left side. Biopsies later demonstrated a subacute or chronic spongiotic dermatitis. The epidermis was acanthotic and showed varying degrees of intercellular edema (spongiosis) with widening of the intercellular spaces; the stratum corneum was thickened and focally compact; the dermis contained a perivascular lymphohistiocytic infiltrate which extended around the superficial and deep vascular plexus. Of note, the biopsies did not show the changes commonly associated with epidermal nevi: alternating ortho and parakeratosis, epidermolytic hyperkeratosis, or acantholytic dyskeratosis. Rather, these clinical and histologic changes in the skin likely represent a form of blaschkitis, an inflammatory skin condition, presenting as papules or vesicles, occurring along the lines of Blaschko (which represent somatically distinct bands of ectodermal migration).

Gastrointestinal: In infancy, she experienced recurrent emesis and diarrhea unresponsive to formula changes. Bloody stools were noted at 10 months of age and watery diarrhea and abdominal pain became persistent. Repeat endoscopic biopsies demonstrated chronic, unspecific inflammation at various sites (most frequently colonic, but also gastric, duodenal, ileal and esophageal regions). Eosinophilic infiltration of the colon was consistently noted. Symptoms were only marginally responsive to treatment with corticosteroids, chronic antibiotics and a severely restricted diet.

Growth disturbances: Growth impairment was reflected by short stature (Z score <−3) and low body weight (Z score −2 to −8), currently 138 cm and 31 kg. Nutritional etiologies were addressed by placement of a G-tube at age 10 with some improvement in growth. Growth hormone was administered for 5 years with moderate benefit. Leg length discrepancy was identified at birth, with left extremity smaller than right in girth and length.

Immunologic: Allergic reactions were observed to enalapril (anaphylaxis), milk (rash), soy (rash) and wheat (rash). She also experienced occasional episodes of dyspnea along with lip and leg angioedema, without an identifiable inciting allergen. Asthma was diagnosed and managed with bronchodilators. Acute phase reactants were noted to be consistently elevated, including ESR and C-reactive protein. Complement (C3 and C4) levels were within reference range. Likewise, quantitative immunoglobulin testing for IgG, IgA, IgE and IgM was within normal limits. Seroconversion after immunization was observed for all vaccinations except varicella virus, hepatitis A virus and hepatitis B virus.
Family history was largely unremarkable, with no family history of consanguinity or gastrointestinal, renal, immunologic or dermatologic disease. Mother, father and older brother are alive and well.

Response to tofacitinib: After 8 weeks of tofacitinib there was complete normalization of acute phase reactants, ESR and C-reactive protein upon laboratory assessment. The patient was previously unable to tolerate dairy, soy and gluten due to severe abdominal pain and diarrhea. After initiation of tofacitinib, she liberalized her diet without restriction and remained asymptomatic, with formed stools and without abdominal pain. At baseline the endoscopic findings included altered vascularity and friable mucosa from rectum to descending colon with microscopic patchy, active colitis with eosinophilic infiltration noted in the ascending colon. After 6 months of treatment on tofacitinib 5mg daily, the colon was grossly normal, with microscopic active colitis and complete resolution of eosinophilia. The dose was further increased to 7.5 mg daily thereafter. The dermatitis significantly improved, as seen in the images. Tofacitinib is 30% renally-excreted and the dose administered was limited due to chronic kidney disease. Presumably, after retransplantation the dose may be escalated with a potentially greater effect. To date, the drug has been very well tolerated with no evidence of adverse effects with close monitoring.

Coronavirus Disease 2019 (COVID-19): Two years after initiation of tofacitinib treatment, the patient developed an acute respiratory infection that was later diagnosed as COVID-19. She was admitted to the hospital where she was treated with azithromycin and hydroxychloroquine and subsequently intubated. Her immunosuppressive treatment regiment, including tofacitinib, was held constant over this time. Unfortunately, despite intensive supportive care, the patient expired 7 days after admission.

Cell lines
U4C cells (JAK1<sup>-/-</sup>) and γ2A cells (JAK2<sup>-/-</sup>) were obtained from S. Pellegrini and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen), GlutaMAX (350 ng/ml; GIBCO), and penicillin/streptomycin (GIBCO). All cell lines expressing ectopic JAK1 and JAK2 variants were generated by lentiviral transduction and were subsequently selected with puromycin and FACS for matched RFP expression. EBV-transformed lymphoblastoid cell lines (EBV-B cells) were generated by infecting PBMCs from healthy controls or the patient with EBV supernatants. EBV-B cells were cultured in RPMI with 10% FBS, 1% glutamine and 1% penicillin/streptomycin. Single-cell clones were isolated by limiting dilution analysis on OP9 feeder cells and expanded in conditioned media. After genotyping by Sanger sequencing, WT/WT and WT/S703I clones were selected.

**METHOD DETAILS**

**Variant analysis**
All samples were collected with informed consent in accordance with IRB-approved protocols (Study ID# IF2349568). For whole-exome sequencing, DNA was isolated (QIAGEN Cat No 69504) from Ficoll-isolated granulocytes from whole blood of the proband and her healthy parents. Library preparation, sequencing (150 bp paired-end reads) and alignment for whole-exome sequencing were performed with the Genewiz exome-sequencing package. Potential disease-causing variants were investigated by Ingenuity Variant Analysis (QIAGEN). High-quality variants were identified by filtering as follows: exclusion of common variants (> 0.1% allele frequencies in public databases); retention of variants in coding regions resulting in substitutions, premature stops, frameshifts or altered splicing; exclusion of variants with CADD scores below MSC thresholds. For recessive models of inheritance, only homozygous or compound heterozygous mutations were assessed. For de novo inheritance, all variants in the parents were excluded, and alleles with known haploinsufficiency, hemizygous, or dominant-negative effects were included. For the validation of WES results, JAK1 was then amplified from PBMC DNA by PCR and Sanger sequenced.

**Digital droplet PCR**
For the determination of mosaic allele fractions, DNA was isolated from bilateral buccal swabs, fractionated blood and a gastrointestinal biopsy specimen in which the epithelial layer was isolated by chemical dissociation. Digital droplet PCR was performed with JAK1 amplification primers, mutation-specific probes (IDT), and with ddPCR Supermix (Biorad 1863026). Amplification and quantification were performed on a QX100 Droplet Digital PCR system (BioRad). Cellular genotypes were estimated with QX100 software (BioRad), assuming heterozygosity.

**Autoantibody Array**
We analyzed the presence of circulating autoantibodies using IgG autoantibody arrays (RayBiotech PAH-AIDG-G1-16). In addition to samples from the patient in this study, plasma from five healthy donors, two patients with systemic lupus erythematosus and Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) were included. All samples were run in duplicate. Before processing, samples were was first clarified and diluted at a ratio of 1:200. Genepix Pro 7.0 software was used to analyze the images.

**Cloning and mutagenesis**
The JAK1 plasmid was obtained from Addgene (Plasmid #23932) in a Gateway-compatible backbone, pDONR 223. Site-directed mutagenesis with specific primers (Quickchange II, Agilent 200521) was performed to obtain the WT coding sequence in the same plasmid, and plasmids encoding the S703I, K908A or S703I/K908A forms were then generated. A control vector containing the luciferase gene was also cloned. Plasmids were then subcloned into a lentivirus-compatible pTRIP-X-IRES-RFP backbone with
puromycin resistance. Pseudotyped lentiviral particles were produced by the transfection of HEK293T cells with pCAGGS-VSV-G, pCMV-Gag/Pol and genes of interest.

**In vitro stimulations**

For the analysis of STAT phosphorylation, cells were stimulated with the indicated doses of recombinant IFN-γ-2b (Intron-A, Merck), IFN-γ, IL-2, IL-4, and IL-6, IL-13 (Biologend) for 15 minutes and then lysed for western blotting. For the analysis of gene induction, cells were stimulated for 8 hours, after which cells were lysed for RNA isolation. For JAK inhibitor treatment, cells were incubated with ruxolitinib (Selleckchem S1378), filgotinib (SelleckchemS7605) or tofacitinib (Selleckchem S5001) at the indicated doses for 4 hours.

**Immunoblotting**

Cells were lysed in RIPA buffer (Thermo Fisher 89900) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling #5872). Lysates were sonicated, centrifuged to remove insoluble complexes, then boiled with NuPage sample buffer (Thermo Fisher NP0007) containing 20 mM DTT. The samples were subjected to gel electrophoresis and semi-dry transfer, and the resulting immunoblots were blocked in 5% BCA, then incubated overnight with primary antibody followed by HRP-conjugated secondary antibodies. Primary antibodies against the following targets were used: GAPDH (Cell Signaling D16H11), STAT1 (Santa Cruz C-111), phospho-STAT1 (Cell Signaling 58D6), phospho-STAT2 (Cell Signaling D3P2P), phospho-STAT3 (Cell Signaling D3A7), phospho-STAT5 (Cell Signaling C11C5), phospho-STAT6 (Cell Signaling 9361), JAK1 (Santa Cruz B3), JAK2 (Cell Signaling D2E12), TYK2 (Cell Signaling D415T), phospho-JAK1 (Cell Signaling 3331), phospho-JAK2 (Cell Signaling 3771), and phospho-TYK2 (Cell Signaling 9321). For the analysis of JAK phosphorylation, lysates were first incubated overnight with antibodies against total JAK protein conjugated to Protein G Dynabeads (Thermo Fisher 10007D). Immunoblotting was then performed as above.

**RT-qPCR**

Cell lines or isolated PBMCs were lysed and RNA was isolated with RNeasy spin columns (QIAGEN 74104). Reverse transcription was performed with the High-Capacity RT Kit (Applied Biosystems 4368814). The resulting cDNA was then subjected to qPCR with the TaqMan Master Mix II with UNG (Thermo Fisher 4440038), on a Roche LightCycler 480, with the following primers/probes: 18S (4318839), MX1 (hs00895608), RSAD2 (hs00369813), SIGLEC1 (hs00988063) IFT1 (hs01911452). The relative expression of each transcript was normalized relative to 18S by the ∆∆Ct method.

**Mass cytometry**

Whole blood was collected, by venipuncture, into sodium heparin vacutainer tubes. For immunophenotyping, blood was immediately stained and processed for mass cytometry. For intracellular staining, whole blood was stimulated by incubation with primary antibody followed by HRP-conjugated secondary antibodies. Primary antibodies against the following targets were used: CD3 (BD 555048), CD4 (BD 555049), CD8 (BD 555045), CD14 (BD 555039), CD19 (BD 555081), PD1 (Biolegend 162501), IFN-γ (Biolegend 101201), TNF-α (Biolegend 110201), and ST2 (Biolegend 175104). For the analysis of JAK phosphorylation, lysates were first incubated overnight with antibodies against total JAK protein conjugated to Protein G Dynabeads (Thermo Fisher 10007D). Immunoblotting was then performed as above.

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Whole blood was collected, by venipuncture, into sodium heparin vacutainer tubes. For immunophenotyping, blood was immediately stained and processed for mass cytometry. For intracellular staining, whole blood was stimulated by incubation with primary antibody followed by HRP-conjugated secondary antibodies. Primary antibodies against the following targets were used: CD3 (BD 555048), CD4 (BD 555049), CD8 (BD 555045), CD14 (BD 555039), CD19 (BD 555081), PD1 (Biolegend 162501), IFN-γ (Biolegend 101201), TNF-α (Biolegend 110201), and ST2 (Biolegend 175104). For the analysis of JAK phosphorylation, lysates were first incubated overnight with antibodies against total JAK protein conjugated to Protein G Dynabeads (Thermo Fisher 10007D). Immunoblotting was then performed as above.
Flow cytometry
Cryopreserved PBMCs from the patient of this study (collected before and after the initiation of tofacitinib treatment), a patient harboring a c.1154C > T p.T385M STAT1 GoF mutation (Vargas-Hernandez et al., 2017), and three unrelated healthy donors were thawed and allowed to rest briefly in complete RPMI medium supplemented with 10% FCS. Cells were immunostained with antibodies in 2% FBS in PBS for 45 minutes. For the panel assessing effector function and activation, cells were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich) for 4 hours at 37°C in the presence of brefeldin A (10 μg/ml, Sigma-Aldrich) and anti-CD107a antibody. For the panels evaluating effector function and transcription factors, cells were then permeabilized with BD Cytofix/Cytoperp (BD Biosciences) or FoxP3 buffer (Tonbo), and were stained with antibody for 45-60 minutes. Activated cells were stained for surface markers for 20-25 minutes after the four hours of incubation. Data were acquired on a FACSArria machine (BD Biosciences) with the capacity to detect 18 fluorescent parameters and exported to FlowJo 10.5.3 (BD Biosciences) for analysis. The frequency of cells positive for each parameter was compared with the mean and standard deviation for three healthy donors analyzed in parallel with the samples from the patient.

IFNAR2 staining was performed on transduced U4C cells surface stained with anti-IFNAR2 antibody (PBL 21385-1) at a 1:100 dilution for 1 hour on ice. Secondary antibody staining was performed with an anti-murine IgG antibody with an Alexa Fluor 647 tag (Thermo Fischer A-21235). Subsequently the cells were stained with a Live/Dead Aqua stain (Thermo L34957) and then fixed in 4% PFA. Data were acquired on a BD Canto II machine (BD Biosciences) and analyzed using FlowJo 10.5.3.

Multiplex ELISA
Plasma was collected by Ficoll isolation from heparinized whole blood and clarified by centrifugation. Circulating cytokine levels were determined in magnetic Lumixx assays with the Bio-Plex Pro Human Inflammation (BioRad 171a001m) and custom Human Cytokine Panel (R&D LXSAHM), according to the manufacturer’s protocol. Samples were quantified on a MAGPIX xMAP Instrument (Lumixx). Cytokine concentrations were quantified by comparison with standard curves and were subsequently translated into Z-scores.

Immunohistochemistry
Immunohistochemistry staining was performed with a Discovery Ultra instrument (Roche), with the staining module of RUO Discovery Multimer V2 (V0.00.0083). Slides were first incubated in blocking agent containing 2% BSA PBS, and Discovery Ultra antibody block (Cat # 760-4204) (Roche). Slides were incubated with primary antibodies for 60 minutes at 37°C. The following primary antibodies were used: anti-phospho-STAT1 (Tyr701) 58D6 (Cell Signaling) and anti-phospho-STAT3 (Tyr 705) D3A7 XP (Cell Signaling) at 1:100 dilution in blocking agent. The slides were then incubated with Omni Map anti-rabbit HRP-conjugated secondary antibody (Multimer HRP) (Cat # 760-4311) (Roche) for 32 minutes. Positive signals were detected with the Discovery ChromoMap DAB Kit (Cat #760-159) (Roche).

Modiﬁed inDrops single cell RNA-Seq targeting JAK1 S703I site
inDrops and related droplet microfluidics singe cell RNA-Seq strategies coencapsulate single “barcode microbeads” and individual cells in droplets. Reverse transcription of polyadenylated (polyA) mRNA incorporates a cellular barcode sequence (different for each bead) into nascent cDNA. After downstream high throughput sequencing, reads can be assigned to individual cells by barcode sequences, thereby enabling expression quantification at single cell resolution. As the cellular barcode is introduced downstream of transcript polyA tails, sequence data is typically restricted to transcript regions immediately proximal to 3’ termini. Because the S703I site is located at position 2402 from 5’ transcript start and 2690 from the 3’ transcript polyA tail, it is not accessible by standard droplet microfluidics single cell RNA-Seq protocols. Therefore, we adapted the inDrops method by generating custom barcode microbeads containing JAK1-specific primers (flanking the S703I site) in addition to polyT primer sequences, enabling more efficient JAK1 target capture and access to the S703I site. Following within-droplet reverse transcription, second strand synthesis and in vitro amplification, samples are split into two parallel library preparations: one for standard polyT-primed libraries, and one for JAK1-targeted libraries. During data processing of resulting high throughput sequencing data, sequence reads from both libraries are assigned to individual cells based on shared barcode sequences. A detailed description of this approach appears below.

JAK1-targeted hydrogel microbeads
Barcoding hydrogel beads were prepared according to established inDrops protocol (Zilionis et al., 2017) with modified primers (Zilionis et al., 2019) with the following modifications. For the second round of split-and-pool primer extension for barcode synthesis, hydrogel beads were added to microplate wells (n = 384) containing oligonucleotide templates for both standard polyT primers and for an additional primer complementary to an S703I-adjacent region of the JAK1 transcript (11.53uM polyT oligonucleotide template, 2.3uM JAK1 oligonucleotide template) Within a given well, both polyT and JAK1 oligonucleotide templates carried identical “barcode 2” sequences, ensuring that extended primers on hydrogels contained matching barcodes. Subsequent exonuclease processing steps included corresponding JAK1 primer complementary blocking oligonucleotides.
JAK1-targeted library preparation

Freshly isolated PBMC were co-encapsulated with JAK1-targeted hydrogel beads. The standard inDrops protocol was followed for reverse transcription, droplet breakage, second strand synthesis and in vitro transcription (IVT) amplification. IVT reactions (20 ul) were then split to two parallel library preparations: 10 ul of IVT product was prepped for polyT-primed gene expression libraries according to the standard inDrops protocol, and 10 ul of IVT product (typically reserved as a “backup” aliquot) was prepped according to a JAK1-targeted protocol as follows. IVT products were reverse transcribed by SuperScript III with a JAK1-specific primer (with 5’ extension containing Illumina adaptor sequence) flanking the S703I site (55C for 1 hr, 70C for 15 min). RT reactions were treated with RNase H (37C for 30 min, 65C for 20 min) to remove RNA template from cDNA heteroduplexes. Following purification on 1.5X Ampure XP beads (Beckman Coulter), JAK1-enriched cDNA was amplified by PCR with Illumina-adapted inDrops primers (KAPA HiFi Master Mix; 2 cycles 98C x 20 s, 55C x 30 s, 72C for 40 s; 16-18 cycles 98C x 20 s, 65C x 30 s, 72C for 40 s; final 72C extension x 5 min). Final JAK1-targeted libraries were purified on 0.8X Ampure XP beads.

Oligonucleotide sequences

doS 5’-BAAAAAAAAAAAAAAAAAAAAANNNNNN [bc2, 8nt] CTGTCTCTTATACACATCTCCGAGCCCACG – 3’
JAK1 oligonucleotide template 5’ - GAGTGTGGCCCATCACAANNNNNN [bc2, 8nt] CTGTCTCTTATACACATCTCCGAGCCCACG – 3’
JAK1 blocking oligonucleotide 5’-GAGTGTGGCCCATCACAANNNNNN [bc2, 8nt] CTGTCTCTTATACACATCTCCGAGCCCACG – 3’

Final “on bead” polyT primer sequence 5’- CGATGACGATAATACGACTACTAGATTGGTGCGGTCGACG[bc1, 8nt] CTGTCTCTTATACACATCTCCGAGCCCACG
GCTCGGAGATGGTA TAAGAGACAG[bc2, 8nt][NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN]
GCTCGGAGATGGTA TAAGAGACAG[bc2, 8nt][NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN]

Second reverse transcription JAK1 primer: 5’- TCGTCGCCAGCUGCAGTGTA TAAGAGACAGCGCATCGAGAATTCAAGTTGCGCAACAG-3’

High Throughput Sequencing

Both standard polyT-primed libraries and JAK1-targeted libraries were pooled together and sequenced in multiplex on the Illumina NextSeq 500 platform with 75-cycle reagent kits in paired-end, dual index configuration:

Read 1 containing transcript/JAK1 data: 61 cycles
i7 read containing cell barcode data: 8 cycles
i5 read containing sample index data: 8 cycles
Read 2 containing cell barcode and unique molecular identified (UMI) data: 14 cycles

Data Processing

FASTQ sequence files were processed with the inDrops.py workflow script (v0.3, https://github.com/inDrops/inDrops). For polyT-primed gene expression libraries, resulting gene x cell matrices and per cell read counts were used for downstream analyses in Seurat (details below), using only “cell-containing droplets” defined by a read count higher than 300-1000 depending on read count evaluation from droplets in the corresponding library batch.

For JAK1-targeted libraries, BAM files (appended with cellular barcode and UMI data) were used to evaluate genotypes at single cell resolution as follows. Reads covering the JAK1 S703I site (chr1:64845519 – 64845521 on the minus strand; human genome reference GRCh38) containing the wild-type sequence (GAT) or S703I (GCT) sequence were quantified per UMI per cellular barcode. If at least 2 and at least 90% of the reads for a given cellular barcode/UMI combination contained the same sequence (wild-type or S703I), then this combination was designated a JAK1 transcript of the given genotype and assigned to the appropriate cellular barcode.

We next used per cell JAK1 WT and S703I transcript counts to assign putative genotypes to individual cells. As cell free RNA in suspension can co-encapsulate with cells thereby generating unwanted background signal in droplet microfluidics scRNA-Seq methods, we aimed to apply a stringent, data driven threshold for genotyping assignment. To evaluate the potential influence of cell free RNA, we quantified the frequency of JAK1 transcripts detected in empty (i.e., cell free) droplets (defined as barcodes with 1000 – 2000 reads in polyT-primed libraries) using the same barcode/UMI strategy described above. We found that more than 99% of empty droplets had less than 3 JAK1 transcripts. Guided by these data, cells were only considered for JAK1 genotype assignment if at least 5 JAK1 transcripts were detected. For JAK1 genotype assignment, individual cells were classified in one of the following four categories: cells that carried the S703I allele were classified as “MUT+” regardless of carrying the wild-type allele; the remainder (S703I allele negative) cells that carried the wild-type allele were classified as “WT+MUTneg” if no S703I transcripts were detected, and as “WT+MUTnonZero” if 1-to-4 S703I transcripts were detected; cells that carried neither the wild-type nor the S703I allele were classified as “WTnegMUTneg.”

Single cell RNA-Seq Gene Expression Analysis

Data from JAK1-targeted inDrops single cell RNA-Seq data were analyzed in conjunction with corresponding single cell RNA-Seq data acquired from the same PBMC specimen on the 10X Genomics Chromium platform. Sequence reads from 10X libraries were processed with the CellRanger software package (10X Genomics) using default parameters. Gene x cell matrices from both
methods were further analyzed with Seurat (v2.3.4) (Butler et al., 2018) in the R statistical framework as follows. For applicable cells in the JAK1-targeted inDrops dataset, JAK1 genotyping information was imported as per cell metadata entries. Genes with detectable expression in fewer than 5 cells per sample were excluded. Cells with detectable expression of fewer than 250 genes or fewer than 1000 UMI counts or greater than 20% UMIs from mitochondrial gene transcripts (measure of cell viability) were removed from further analysis. Gene expression data were log normalized and scaled (regressing out effects based on total UMI counts and mitochondrial gene expression). All genes with detectable expression in both inDrops and 10X datasets were used for CCA dimensionality reduction (20 dimensions). CCA dimension scores were aligned between datasets using the AlignSubspace function. Aligned data were log-normalized and scaled as above. Graph-based clustering and tSNE visualization was performed with the FindClusters and RunTSNE functions with default settings (using the first 19 CCA dimensions, based on examination of distinct expression patterns with the DimHeatmap function). Marker genes for individual clusters were identified with the FindMarkers() function in Seurat (parameters: min.pct = 0.1, only.pos = TRUE, logfc.threshold = log(1.5), min.diff.pct = 0.1, pseudocount.use = 0.1). Briefly, up to 100 cells were randomly selected from each cluster, and each cluster was contrasted against all others for differential gene expression. Significant genes (p < 0.05) were ranked by log2 fold-change; the top 25 genes for each cluster, as well as a select list of additional genes of interest, were selected as marker genes for supervised cluster annotation (Table S3). Expression patterns were further evaluated on tSNE plots. Select clusters were merged based on shared expression patterns as indicated.

Gene expression differences were assessed for cells assigned JAK1 genotype classifications of either MUT+ (S703I) or WT+MUTneg (WT). Plasmablasts and dendritic cells were excluded from analysis due to insufficient cells assigned to both genotype groups. Apparent cellular doublets and cells in the platelet cluster were also excluded. Differential gene expression testing was performed on genes detected in at least 20% of cells in any cell type cluster (of either JAK1 genotype group) with edgeR (v.3.12.1) (Robinson et al., 2010), including modifications included for single cell RNA-Seq data as described (Soneson and Robinson, 2018). A linear model including factors for cellular gene detection rate, cell type, JAK1 genotype group and an interaction term (cell type cluster and JAK1 genotype group), was fit with the glmQLFit function. Differential gene expression testing (WT versus S703I) was tested across all cell types with the glmQLFtest function, with significance thresholds set at FDR 0.05. In addition, gene set enrichment testing for Molecular Signatures Database Collections H, C2- CPC, C3-TFT and C7 collections (http://software.broadinstitute.org/gsea/msigdb), supplemented with two additional interferon stimulated gene sets (Rosenberg et al., 2018) was performed with CAMERA (Wu and Smyth, 2012) and the linear model described above. Only gene sets with 5 or more genes in the fitted model were included in the analysis.

**Single Cell qPCR Transcript Genotyping**
PBMCs from a healthy donor carrying a heterozygous SNP in JAK1 (rs2230587) were isolated by Ficoll gradient. Cells were stained with antibodies against CD3, CD19, CD14 and CD56 (Biolegend), and 100 single cells were FACSSorted into single cell lysis buffer (Ambion 4458235). Following DNase treatment, cDNA was generated using SuperScript VLo RT kit (ThermoFisher 11754050). A linear preamplification was then performed using JAK1 primers (gtcctctggatctcttcatgca, gctgtttggcaactttgaatttcc) and primers to a negative control gene NACA (cccaggcaaccacacaac, ccgactctgttttgctttactgact). Using qPCR with custom TaqMan genotyping primers (above) and allele-specific probes (JAK1 aaggacatc[g/a]cttttc; agcagctgaaat[T/C]gatgaa) that were individually fluorescently tagged (VIC and FAM), allelic ratios were determined by endpoint genotyping. Quantification was carried out by interpolation from a standard curve of oligonucleotide standards.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Data are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated in the legend for each figure. Statistical parameters including the methods implemented, corrections for multiple comparisons, exact values of n, identity of replicates, definitions of center and dispersion and statistical significance are reported in the Figure Legends when necessary. Statistical testing for the analysis of single cell RNA sequencing is discussed in the corresponding section of the Method Details. All statistical tests were calculated in the R statistical framework or GraphPad PRISM.