

An N-of-1 Trial of Itacitinib for a Patient with Aplastic Anemia Associated with a Gain-of-Function Variant in STAT1

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1 **An N-of-1 Trial of Itacitinib for a Patient with Aplastic Anemia Associated with a Gain-of-**
2 **Function Variant in STAT1**

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28

29 **Abstract**

30 An 18-year-old man presented with aplastic anemia, and exome sequencing identified a germline
31 gain-of-function variant in the gene *STAT1*. Treatment with itacitinib, an investigational selective
32 Janus Kinase 1 (JAK1) inhibitor, resulted in prompt recovery of hematopoiesis. An exhausted CD8+
33 T cell population and myeloid populations enriched for an interferon- γ signature correlated with
34 disease activity. Patient bone marrow sections displayed increased phospho-STAT1 staining, as did
35 other idiopathic aplastic anemia cases, suggesting a shared pathophysiologic mechanism. This study
36 describes the success and mechanism of a molecularly targeted therapy with potential implications for
37 the treatment of aplastic anemias and other autoimmune disorders.

38 **Introduction**

39 An 18-year-old man presented with 3 weeks of pallor, weakness, and dyspnea. On exam, the patient
40 was pale but well appearing with a BMI of 17. He had buccal aphthous ulcers and thrush on the soft
41 palate. His peripheral blood laboratory values were notable for pancytopenia with a hemoglobin level
42 of 3.9 g/dL (reference range 12-16) without signs of hemolysis, a white blood cell count nadir of 350
43 cells/ μ l (reference range 4,500-11,000), an absolute neutrophil count nadir of 210 cells/ μ l (reference
44 range 1,800-7,700), and a platelet count of 118,000/ μ l (reference range 140,000-430,000). He was
45 transfused red blood cells, and a bone marrow biopsy demonstrated hypocellularity (20-30%
46 cellularity) with a myeloid predominance, absence of erythroid precursors, and decreased
47 megakaryocytes. An infectious and rheumatologic workup was unrevealing (**Fig. S1**). Testing for
48 paroxysmal nocturnal hemoglobinuria, Fanconi anemia, and telomere shortening were negative. Bone
49 marrow panel mutational testing, karyotypic analysis, and T-cell receptor spectrotyping were all within
50 normal limits. Though platelets were relatively preserved, based on criteria of peripheral neutrophils
51 <500/mL, reticulocytes < 1%, and bone marrow hypocellularity, a diagnosis of severe aplastic anemia
52 was made.¹

53 His past medical history was notable for recurrent severe oral aphthous ulcers since childhood, for
54 which he took prednisone 10mg during flares. Given his thrush, we prescribed nystatin oral
55 suspension which he took as needed. His paternal grandmother, father, and other family members
56 suffered similar oral ulcers since youth, and construction of a pedigree suggested an autosomal
57 dominant pattern of inheritance (**Fig. 1A**). His father also suffered from thrush, but additional family
58 history beyond these clinical features was unable to be obtained. No other family members were
59 known to have aplastic anemia.

60 **Results**

61 Molecular Evaluation

62 Aplastic anemia is a rare disorder characterized by pancytopenia due to bone marrow failure. It can
63 be caused by direct damage (e.g. toxins or radiation), rare genetic syndromes, infection, or, most
64 commonly, idiopathic autoimmune T cell attack of hematopoietic cells.² Standard-of-care therapy
65 includes allogeneic stem cell transplantation or intensive immunosuppression.

66 Given the autosomal dominant inheritance pattern of the patient's aphthous lesions and unclear
67 etiology of his aplastic anemia, we performed exome sequencing of both patient and father. A
68 heterozygous variant (c.800C>T, p.Ala267Val) in the coiled-coil domain of *STAT1* was identified in
69 both the patient and his father and confirmed by Sanger sequencing (**Fig. 1B**).

70 *STAT1* is a transcription factor downstream of JAK signaling, a pathway critical to hematopoiesis,
71 immunity, and development.³ More than 50 cytokines and growth factors, including interferons, signal
72 through this pathway by binding to cognate receptors and effecting dimerization. Dimerization
73 activates receptor-bound JAKs which then phosphorylate and activate STATs. STATs form either
74 homo- or hetero-dimers, translocate into the nucleus, bind specific DNA sequences, and activate
75 discrete transcriptional programs.

76 Gain of function (GOF) variants in *STAT1* cause an autosomal dominant syndrome with a spectrum of
77 autoimmune features and near complete penetrance of mucocutaneous candidiasis.^{4,5} More than 400

78 patients with more than 100 different STAT1 GOF variants have been reported.^{4,6,7} Aplastic anemia
79 has been described, as have numerous other autoimmune cytopenias, and severe aphthous
80 stomatitis is frequent.^{4,6,8,9} GOF variants lead to hyper-phosphorylation of STAT1 in response to
81 stimulation, increasing STAT1-dependent transcription.^{7,10,11} The p.Ala267Val variant identified in our
82 patient is absent from large population databases, but has been identified in more than 10 individuals
83 in multiple families with chronic mucocutaneous candidiasis, and shown to have a GOF effect.^{6,12} We
84 thus considered the p.Ala267Val variant as causal for our patient's recurrent aphthous ulcers, thrush,
85 and aplastic anemia.

86 Itacitinib Trial

87 As STAT1 activation is heavily dependent on JAK signaling, the JAK inhibitors ruxolitinib and
88 tofacitinib have been trialed in STAT1 GOF patients. Successful and unsuccessful cases have been
89 reported for the treatment of alopecia, diabetes, thrush, fungal infections, and autoimmune
90 cytopenias.^{8,13-18} Ruxolitinib and tofacitinib inhibit both JAK1 and JAK2 with the potential for anemia
91 and thrombocytopenia, since JAK2 is downstream of the erythropoietin and thrombopoietin receptors.
92 In considering a JAK inhibitor, we thus sought to avoid JAK2 inhibition.

93 The investigational drug itacitinib is a potent JAK1 inhibitor with an IC₅₀ of 3.2 nM and selectivity for
94 JAK1.¹⁹ Itacitinib has been studied in myeloproliferative neoplasms, graft-versus-host disease, and
95 autoimmune disorders.

96 The patient remained dependent on red blood cell transfusions, and he was thrice admitted to the
97 hospital for febrile neutropenia. Preparations were underway for hematopoietic stem cell
98 transplantation when, based on genotyping results, we enrolled the patient in a single patient
99 expanded use trial of itacitinib (<https://clinicaltrials.gov/ct2/show/NCT03906318>). On day 169 after
100 presentation, he received his weekly transfusion and initiated itacitinib 300mg daily. The following
101 week, and for all subsequent visits after the initiation of itacitinib, laboratory values demonstrated
102 resolution of neutropenia and anemia, and the patient became red blood cell transfusion independent
103 (**Fig. 1C**). A subsequent bone marrow biopsy demonstrated a return of trilineage hematopoiesis.

104 Increased STAT1 signaling as measured by phospho-STAT1 (pSTAT1) staining, which had been
105 present at time of diagnosis, resolved after treatment (**Fig. 1D**). The patient's thrush and oral ulcers
106 persisted but at significantly decreased frequency and severity. Clinically, fatigue and weight loss
107 resolved, and he returned to schooling. He completed 20 months of itacitinib therapy without any
108 adverse events before electing to self-discontinue. Aplastic anemia has not recurred.

109 Immunological Profiling

110 To understand the cellular mechanisms involved in the pathogenesis and resolution of this patient's
111 aplastic anemia, we performed comprehensive immunophenotyping. To confirm STAT1 GOF activity,
112 we used phospho-CyTOF to measure phospho-STAT1 in monocytes after stimulation with interferon- γ
113 at timepoints pre- and post-itacitinib treatment. At all timepoints, the patient's monocytes had higher
114 levels of pSTAT1 than healthy control. Post-itacitinib, pSTAT1 levels decreased, though not to the
115 level of healthy control (**Fig. 2A**).

116 The largest studies of STAT1 GOF patients have demonstrated increased frequencies of T_H1 and
117 decreased frequencies of T_H17 T cells.^{20,21} Increased signaling through STAT1 biases T cell
118 differentiation into the T_H1 phenotype, which is characterized by secretion of interferon- γ . T_H1
119 differentiation comes at the expense of STAT3-mediated T_H17 differentiation, which is characterized
120 by secretion of IL-17A and cytokines critical for immunity to mucocutaneous *Candida*. It is
121 hypothesized that JAK inhibition may restore the balance between T_H1 and T_H17 differentiation by
122 dampening STAT1 signaling.⁸

123 Interrogating the T_H1 and T_H17 pathways, we compared plasma cytokine levels pre- and post-
124 treatment with itacitinib. This identified significant decreases in the T_H1 cytokines interferon- γ and
125 IL-12p40 following therapy, but no significant shifts in the T_H17 cytokines IL-17A or IL-17F (**Fig. 2B**).
126 Of note, elevated serum interferon- γ has been described in aplastic anemia.²²

127 Examining CD4+ T cells by post-stimulation intracellular cytokine staining, we found a near absence
128 of IL-17A-producing CD4+ T cells (T_H17) in the patient compared to healthy controls; this did not

129 change with treatment (**Fig. S2B**). Frequencies of interferon- γ -producing CD4⁺ T cells (T_H1) did not
130 differ between healthy controls, the patient pre-itacitinib, or the patient post-itacitinib (**Fig. 2C**). Thus,
131 we did not observe evidence of itacitinib restoring a balance between STAT1-mediated T_H1 and
132 STAT3-mediated T_H17 frequencies. Moreover, while we observed other peripheral blood mononuclear
133 cells (PBMC) abnormalities including an absence of T_{reg} cells, elevated frequencies of memory CD4⁺
134 and CD8⁺ T cells, and reduced frequencies of T follicular helper (T_{FH}) cells compared to healthy
135 controls (**Fig. S2A**), none of these frequencies changed significantly with itacitinib treatment.

136 Surprisingly, the patient's pre-itacitinib CD8⁺ T cells produced significantly less interferon- γ than
137 healthy controls in vitro (**Fig. 2D**). After treatment with itacitinib, CD8⁺ interferon- γ production
138 increased but not to the level of healthy controls. Pre-itacitinib memory CD8⁺ T cells also had a
139 striking increase in expression of the activation marker Programmed Death 1 (PD-1), which
140 decreased with itacitinib treatment (**Fig. 2E**). Thus PD-1⁺ CD8⁺ T cells—which are defective in their
141 ability to secrete interferon- γ upon stimulation in vitro—closely correlate with aplastic anemia disease
142 activity.

143 Taken together, these findings suggest a model whereby during aplastic anemia, the patient's CD8⁺
144 T cells are chronically activated against hematopoietic progenitors in vivo leading to an exhausted
145 phenotype characterized by impaired ability to secrete interferon- γ in vitro and increased PD-1
146 expression. By inhibiting JAK1-driven activation, itacitinib relieved CD8⁺ T cell exhaustion. Consistent
147 with our findings, elevated frequencies of activated CD8⁺ T cells have been described in aplastic
148 anemia.²³ We did observe the seemingly paradoxical finding of increased serum interferon- γ (**Fig. 2B**)
149 yet decreased interferon- γ secretion by CD8⁺ T cells in vitro (**Fig. 2D**). One interpretation of these
150 results is that these CD8⁺ T cells are chronically secreting interferon-gamma in vivo, yet their
151 exhausted phenotype reduces their capacity to secrete interferon-gamma in vitro upon stimulation.²⁴
152 To explore the in vivo unstimulated cytokine effector function of these cells, we performed single-cell
153 transcriptional analyses.

154 Single-Cell Gene Expression Analysis

155 To understand the transcriptional programs governing our patient's aplastic anemia, we performed
156 single-cell transcriptional sequencing (scRNA-Seq), comparing PBMCs from healthy controls and our
157 patient at timepoints before and after itacitinib. Unsupervised clustering demonstrated that healthy,
158 pre-itacitinib, and post-itacitinib cells represent distinct clusters. Post-itacitinib samples clustered
159 between healthy and pre-itacitinib samples, suggesting progression from disease towards the healthy
160 state (**Fig. 3A**).

161 We then separately subclustered T cell-containing and myeloid populations for further analysis (**Fig.**
162 **S3**). T cell-containing cells from the patient and healthy controls subclustered into a CD8+ population,
163 NK population, and mixed CD4+ and CD8+ memory and naïve populations. Given the activated but
164 dysfunctional state of our patient's CD8+ T cells, we scored all CD8+ T cells for cytotoxicity, cytokine
165 effector function, and exhaustion (**Table S1**). Each of these scores were elevated in the patient's pre-
166 itacitinib cells compared to healthy controls, confirming their cytotoxic and exhausted phenotype (**Fig.**
167 **3B**). After treatment, exhaustion and cytokine effector scores decreased. Our finding of increased
168 transcriptional cytokine effector scores in the patient's pre-itacitinib CD8+ T cells supports the
169 hypothesis that these cells secrete higher levels of cytokines such as interferon- γ in vivo, despite their
170 reduced capacity to secrete interferon- γ in vitro upon stimulation.

171 Subclustering of myeloid populations identified five populations (**Fig. 3C**). Of note, "activated CD14+
172 monocytes" and "C1Q+ monocytes" were found virtually exclusively in the patient's and not in the
173 healthy controls' cells. Both of these subclusters expressed STAT1 activation-induced genes such as
174 the complement genes *C1QC*, *C1QB*, *C1QA* or the interferon-inducible genes *FAM26F* or *GBP1*,
175 respectively (**Fig. S3**). Type I interferons (α , β , and others) and Type II interferon (only γ) induce
176 overlapping but distinct transcriptional signatures. Since monocytes express high levels of the
177 interferon- γ receptor, we scored each myeloid cell to assess for interferon exposure (**Table S1**). Both
178 Type I and II interferon scores in pre-treatment monocytes were higher than healthy control or post-

179 itacitinib monocytes. Within pre-itacitinib monocytes, Type II scores were higher than Type I,
180 suggesting a primarily interferon- γ induced state ($p < 10^{-10}$, Wilcoxon rank-sum).

181 Single-Cell Epigenetic Analysis

182 To understand the epigenetic effects of the STAT1 GOF variant and itacitinib treatment, we performed
183 single-cell Assay for Transposase-Accessible Chromatin with Sequencing (scATAC-seq). Within
184 effector CD8+ T cells, we saw increased accessibility at the PD-1 locus, consistent with our
185 observation of increased PD-1 protein expression (**Fig. 2E**), and this accessibility decreased after
186 treatment (**Fig. 3D and S4**).

187 Genome-wide, STAT1 motif accessibility pre-itacitinib was increased when compared to healthy
188 controls or post-itacitinib samples (**Fig. 3E**). These studies suggest that changes in accessible
189 chromatin correlate with STAT1-mediated autoimmunity and can be reversed with itacitinib.

190 pSTAT1 Bone Marrow Immunostaining

191 Immunostaining our patient's bone marrow sections, we found increased pSTAT1 staining pre-
192 itacitinib, and this STAT1 activity resolved following itacitinib therapy (**Fig. 1D**). To determine whether
193 similar STAT1 dysregulation exists in patients with idiopathic (non-STAT1-mutated) aplastic anemia,
194 we analyzed bone marrow sections from other aplastic anemia patients. Compared to healthy donor
195 marrow in which no staining was detected, three of four marrows exhibited positive pSTAT1 staining
196 (**Fig. 4**). This raises the exciting possibility that STAT1 activation is a feature of a subset of aplastic
197 anemias for which pSTAT1 is a potential biomarker.

198 **Discussion**

199 Here we present a man with a history of oral ulcers inherited in an autosomal dominant pattern who
200 developed aplastic anemia. Exome sequencing identified a pathogenic GOF mutation in *STAT1*.
201 Molecular inhibition of JAK1, a kinase upstream of STAT1 activation, resulted in resolution of the
202 patient's aplastic anemia.

203 Longitudinal immunophenotyping of samples unperturbed by confounding immunosuppressive
204 therapy revealed an expanded, activated, cytolytic, and exhausted memory CD8+ T cell population
205 that correlated with disease activity, as did plasma levels of interferon- γ . After itacitinib treatment,
206 these abnormalities improved, with parallels to mouse and human models of idiopathic aplastic
207 anemia.^{23,25}

208 To our knowledge, this represents the first report of a targeted therapy for the treatment of
209 autoimmune aplastic anemia. This case also marks the first report of itacitinib for use in a primary
210 immunodeficiency. Given the high frequency of cytopenias in primary immunodeficiencies, itacitinib's
211 JAK1 selectivity may be of particular clinical benefit.^{26,27}

212 Establishing the causality of a therapy in a single-patient trial is challenging, but the co-incident timing
213 of itacitinib with near immediate hematopoietic recovery after 6 months of transfusion-dependence, as
214 well as the low expected rate of spontaneous recovery in aplastic anemia,² are evidence in favor of a
215 therapeutic effect of itacitinib in this case.

216 While STAT1 GOF is a rare condition, aplastic anemia is more common. Aplastic anemia can be
217 triggered by a spectrum of host and environmental factors,² but our patient's immunophenotypic
218 similarities to other cases of aplastic anemia raise the possibility of a shared downstream
219 pathophysiology for which JAK inhibitor therapy could be effective. Additionally, our finding of
220 increased bone marrow pSTAT1 staining in a majority of aplastic anemia cases may be a useful
221 biomarker to identify candidate patients. We hope these results spur larger trials to answer these
222 questions, particularly given the relative tolerability of JAK inhibitors compared to current standard
223 treatment modalities including hematopoietic stem cell transplantation or medical
224 immunosuppression.

225 **Online Methods**

226 Please see separate attachment for methods section.

227

228

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237 measuring plasma cytokine levels and phospho-CyTOF analysis.

238 Author Contributions

239 J.M.R. conceived the study and designed the experiments with D.B.S. J.M.R., T.H., J.M.P., C.A.L.,
240 L.S.L., and L.R.M. performed the experiments and analyzed the data. C.A.T. and H.L.R performed the
241 exome sequencing. B.B., A.K.S., Y.B.C., S.M.F., and D.B.S supported the studies and reviewed the
242 manuscript.

243 Competing Interests

244 Y.B.C. reports consulting fees from Incyte. All other authors declare no competing interests.

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Figures

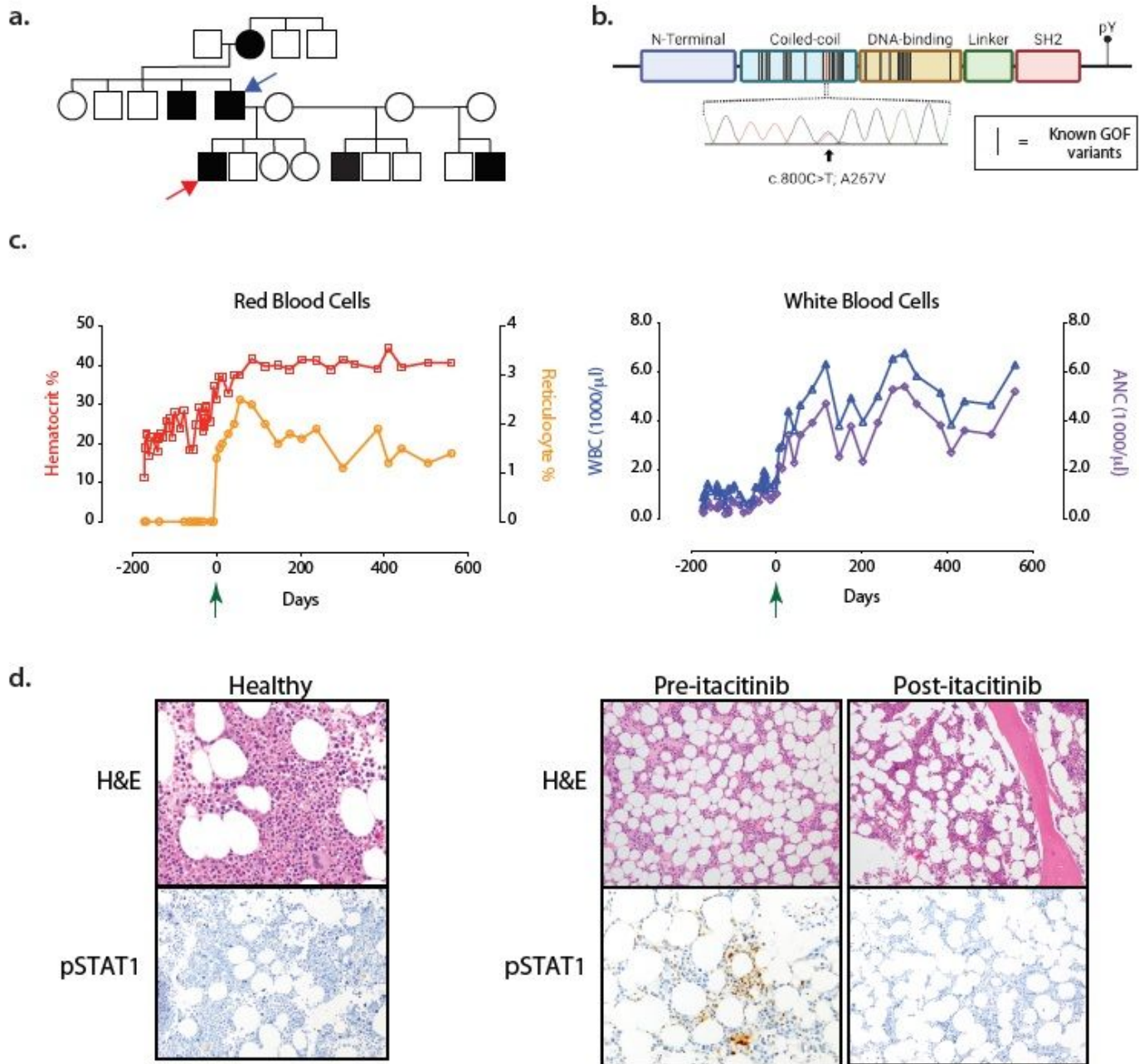


Figure 1

Clinical presentation and response to itacitinib. a. Family pedigree. Males and females are denoted by squares and circles, respectively. The phenotype of severe oral ulcers is demonstrated by shaded symbols. The patient and his father, both heterozygous for the A267V mutation, are noted by the red and blue arrows, respectively. b. Schematic of the domains of the STAT1 protein and its tyrosine phosphorylation site (pY). Black bars represent known STAT1 GOF mutations, the red bar represents the patient's mutation, and Sanger sequencing of the mutation is shown. c. Peripheral blood laboratory

values and response to itacitinib. Hematocrit and reticulocytes are plotted (left). White blood cell (WBC) and absolute neutrophil counts (ANC) are plotted (right). Green arrow on day 0 indicates the start date of itacitinib. d. Bone marrow hematoxylin and eosin (H&E) and phosphorylated STAT1 (pSTAT1) staining are shown for a healthy donor (left) and the patient (right). Sections from the patient pre-itacitinib and post-itacitinib at two separate time-points are shown.

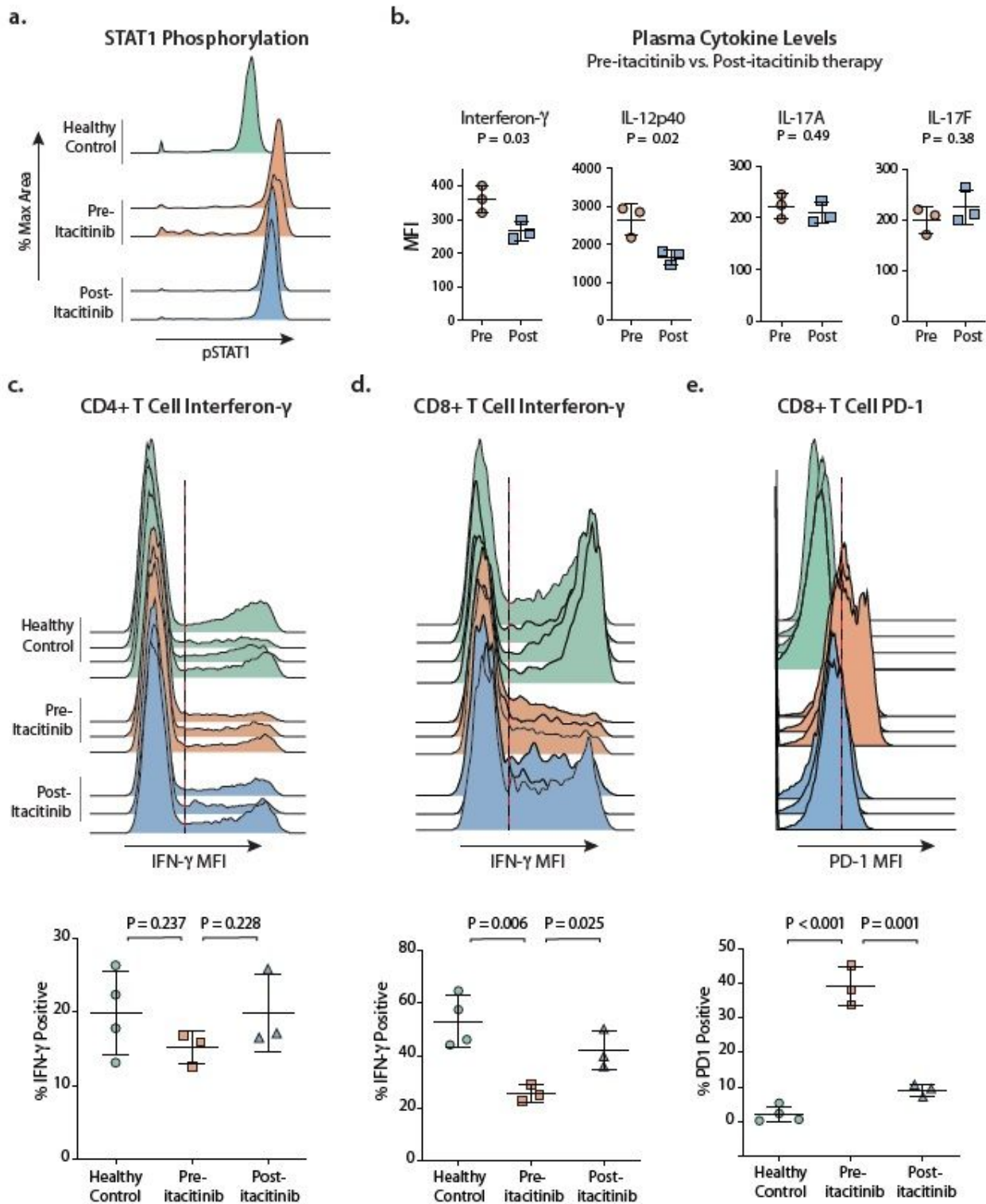


Figure 2

Immunophenotyping. a. Phospho-CyTOF histograms pSTAT1 levels after stimulation in vitro with interferon- γ . Shown are pSTAT1 levels in monocytes from a healthy control and four different time points from the patient pre- and post-itacitinib treatment. b. Plasma cytokine levels measured in units of mean fluorescent intensity (MFI) from the patient at six time points pre- and post-itacitinib. Each sample was measured in duplicate. c. CD4+ memory T cells (CD45RO+) are shown both as histograms and the gated percent of cells secreting IFN- γ . d. CD8+ memory T cells (CD45RO+) are shown both as histograms and the gated percent of cells secreting IFN- γ . e. Programmed Death 1 (PD-1) expression levels on CD8+ T cells are shown as histograms and as the gated percent of cells expressing PD-1. (c.– d.) PBMCs were stimulated in vitro with PMA/ionomycin and stained for intracellular cytokines. (c.– e.) Healthy control n=4, pre-itacitinib n=3, post-itacitinib n=3. (b.– e.) Statistical analyses performed using unpaired t-test.

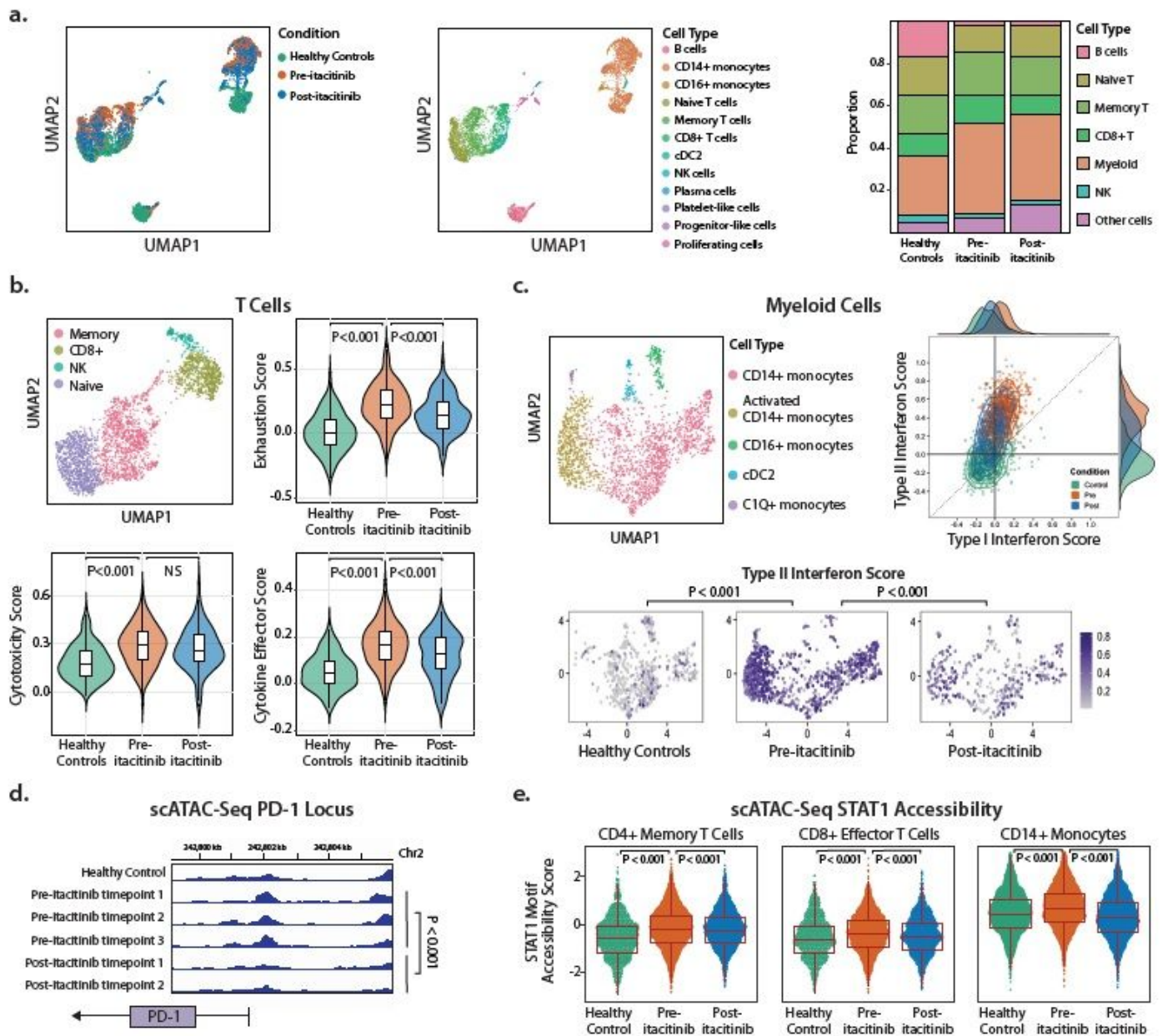


Figure 3

Single-cell transcriptional and epigenetic profiling. a. Unsupervised clustering of all cells by scRNA-Seq expression profiling in Uniform Manifold Approximation and Projection (UMAP) space. Cells are colored by condition (left) or cell type (middle). n=4 different healthy control samples, n=3 pre-itacitinib time points, and n=3 post- itacitinib time points are included. Stacked bar plot shows cell proportions by condition (right). “Myeloid” includes CD14+ monocytes, CD16+ monocytes, and type 2 conventional dendritic cells (cDC2). “Other” cells include plasma cells, platelet-like cells, progenitor-like cells, and proliferating cells. b. Sub-clustering of T cell-containing populations (top left). The CD8+ T cell population (olive green) was then scored for exhaustion, cytotoxicity, and cytokine effector function and plotted by condition. c. Sub-clustering of myeloid populations (top left). Each myeloid cell was scored and plotted (top right) by its Type I interferon score (x-axis) and Type II interferon score (y-axis). Histograms of each of these scores by condition are shown on the plot edge. Each myeloid cell was then plotted by condition and colored by its Type II interferon score (bottom figures). d. scATAC-seq accessibility peaks at the PD-1 locus in healthy control and the patient’s PBMCs at five time points. e. scATAC-seq genome-wide accessibility at STAT1 binding motif sites. Across cell types and conditions as labeled, each cell was scored by STAT1 accessibility. P values calculated using Wilcoxon rank-sum test.

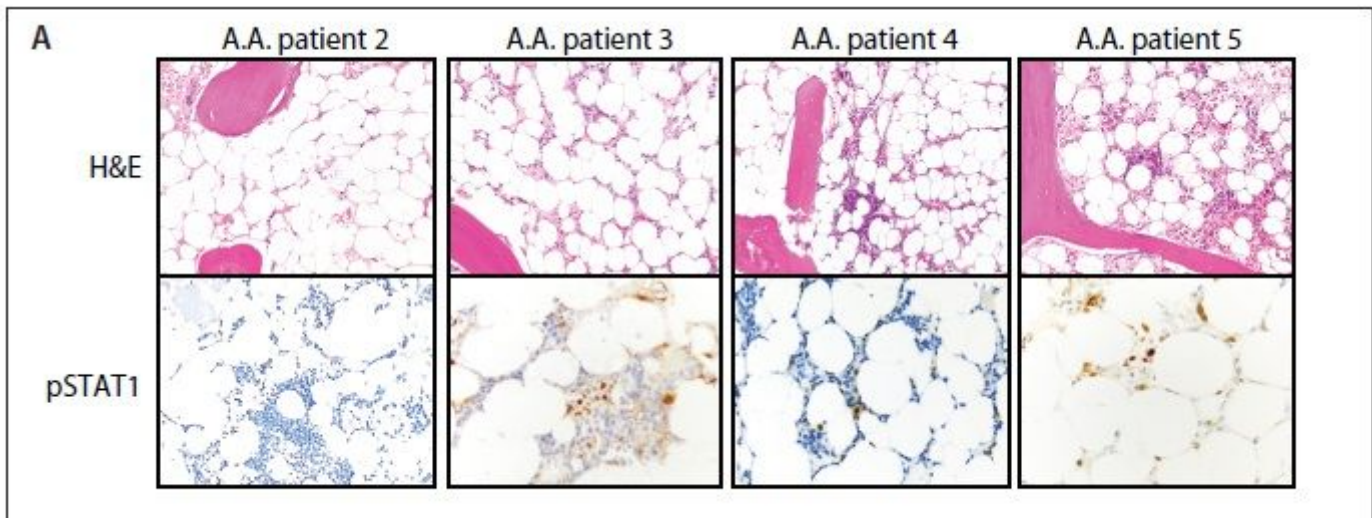


Figure 4

Bone marrow STAT1 activation in historical aplastic anemia cases. Bone marrow hematoxylin and eosin (H&E) and phosphorylated STAT1 (pSTAT1) staining are shown for four historical patients with idiopathic aplastic anemia (A.A.) at the time of their diagnosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [STAT1Methods.pdf](#)
- [TableS1.xlsx](#)

- [SupplementalFigures.pdf](#)