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# JAK2$^{V617I}$ results in cytokine hypersensitivity without causing an overt myeloproliferative disorder in a mouse transduction-transplantation model

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## Abstract

A germline *JAK2*$^{V617I}$ point mutation results in hereditary thrombocytosis and shares some phenotypic features with myeloproliferative neoplasm, a hematologic malignancy associated with a somatically acquired *JAK2*$^{V617F}$ mutation. We established a mouse transduction-transplantation model of *JAK2*$^{V617I}$ that recapitulated the phenotype of humans with germline *JAK2*$^{V617I}$. We directly compared the phenotype of *JAK2*$^{V617I}$ mice with *JAK2*$^{V617F}$ mice. The *JAK2*$^{V617I}$ mice displayed increased marrow cellularity with expanded myeloid progenitor and megakaryocyte populations but this phenotype was less severe than in *JAK2*$^{V617F}$ mice. *JAK2*$^{V617I}$ resulted in cytokine hyper-responsiveness without constitutive activation in the absence of ligand whereas *JAK2*$^{V617F}$ resulted in constitutive activation. This may explain why *JAK2*$^{V617I}$ produces a mild myeloproliferative phenotype in the mouse model as well as in humans with germline *JAK2*$^{V617I}$ mutations.

## INTRODUCTION

A single gain-of-function somatic point mutation in the Janus kinase 2 (*JAK2*) gene is present in the majority of patients with Philadelphia-negative myeloproliferative neoplasm (MPN)[1–5]. JAK2 is a cytoplasmic tyrosine kinase which is critical in intracellular signaling by cytokine receptors such as erythropoietin (Epo), thrombopoietin (Tpo), interleukin-3 (IL-3), granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). The *JAK2*$^{V617F}$ mutation results in a constitutively active JAK2, with continual activation of downstream intracellular signaling cascades[6–8]. Although there is a familial predisposition to acquire MPN[9,10] the *JAK2*$^{V617F}$ mutation is somatically acquired in a hematopoietic stem cell. However, a germline *JAK2*$^{V617I}$ mutation has recently

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**AUTHOR CONTRIBUTIONS:**

SAB, SJM, SBL, and AGF performed experiments, analyzed data, and wrote the manuscript. HYL, TKN, LRR and AA performed experiments, analyzed data, and edited the manuscript. BJD provided research guidance and edited the manuscript.

been identified in a family with hereditary thrombocytosis[11,12]. The ability of *JAK2*$^{V617I}$ to confer cytokine independence had previously been shown in Ba/F3 cells randomly mutated at position 617 of JAK2[13]. Like acquired MPN, family members with germline *JAK2*$^{V617I}$ have thrombocytosis and megakaryocytic hyperplasia in the marrow with increased risk of thrombosis. But unlike acquired MPN, individuals with the *JAK2*$^{V617I}$ germline mutation do not develop a fibrotic bone marrow, splenomegaly, or transform to acute leukemia. Why germline *JAK2*$^{V617I}$ recapitulates some aspects but not others of the MPN phenotype in humans is unclear. To delineate the differences between *JAK2*$^{V617F}$ and *JAK2*$^{V617I}$ we compared the phenotype of mice with hematopoietic cells expressing *JAK2*$^{V617F}$ or *JAK2*$^{V617I}$.

## METHODS

### Bone Marrow Transplantation

C57B/6 mice were purchased from Jackson Labs. Retroviral infection and transplantation was performed as previously described[14]. All mouse work was performed with approval from the Oregon Health & Science University and UC Irvine Institutional Animal Care and Use Committee.

### Standard flow cytometry

The following antibodies were used for identification of mature cell populations: CD41 PE (MWReg30, BD Biosciences), CD42d (1C2, Biolegend) CD11b APC (M1/70, BD Biosciences), Gr-1 PerCPCy5.5 (RB6-8C5, ebioscience), TER119 APC (TER119, BioLegend). For hematopoietic progenitor populations the following antibodies were used: APC lineage (Lin) markers (CD3, KT31.1; CD4, GK1.5; CD8, 53–6.7; B220, 6B2; Mac-1, M1/70; Gr-1, 8C5; and TER119, all from BD Biosciences), c-kit APC-Cy7 (2B8, BD Biosciences), CD34 PE (RAM34, BD Biosciences), CD16/32 PE-Cy7 (2.4G2, BD Biosciences), and Sca-1 Pacific Blue (D7, BioLegend). LKS, CMP, GMP, and MEP are defined as in[15]. Cells were analyzed using an Aria III flow cytometer (BD Biosciences). Data was analyzed using FlowJo software (Treestar).

### Phosflow

Cells were stimulated with 0.1, 0.2, or 1 ng/ml of GM-CSF for 15 min at 37°C. Following stimulation, cells were fixed with paraformaldehyde and permeabilized in methanol. Samples were stained with PE- pSTAT3 (pY694) and A647- pSTAT5 (pY701) (BD Biosciences) along with cell surface markers. Cytobank.org was used to analyze data.

### Methylcellulose Colony Formation Assays

For Figure 2D $1\times10^6$ spleen cells were plated in 1.1ml methylcellulose semi-solid media (M3231, StemCell Technologies) supplemented with 100ng/ml mSCF, 10ng/ml mIL-3 (peprotech) and 3U/ml hEpo (Procrit, Amgen) in triplicate. Colonies were enumerated after 12 days in culture. For Figure 2F GFP$^+$ progenitors (lin$^{neg}$, c-kit$^+$, Sca-1$^-$) were sorted by flow cytometry and plated at a concentration of 1000 cells per 1.1 ml of methylcellulose in triplicate (M3231, StemCell Technologies) supplemented with 100ng/ml mSCF, 10ng/ml mIL-3, 50ng/ml mTPO (peprotech), and 3U/ml hEPO. Plates were examined at 7 days of

culture and scored by visual morphology. The morphology of cells in the individual colonies was confirmed by cytospin with Giemsa staining.

## RESULTS AND DISCUSSION

### *JAK2*$^{V617I}$ expression induces a mild MPN phenotype

Lethally irradiated C57B/6 mice were transplanted with equal numbers of bone marrow cells expressing *JAK2*$^{V617F}$, *JAK2*$^{V617I}$, or empty MSCV-IRES-GFP (MIG) vector[16]. As expected, JAK2$^{V617F}$ mice developed erythrocytosis and leukocytosis. However, JAK2$^{V617I}$ mice had peripheral blood counts similar to empty vector mice (Fig 1A–C). To rule out the possibility that the phenotypic differences we observed in *JAK2*$^{V617F}$ versus *JAK2*$^{V617I}$ mice was not simply due to lower expression of JAK2 in *JAK2*$^{V617I}$ mice, we confirmed equivalent expression of JAK2 in *JAK2*$^{V617F}$ and *JAK2*$^{V617I}$ mice by quantitative RT-PCR (data not shown) and Western Blot (Supplemental Figure 1). The lack of thrombocytosis in the *JAK2*$^{V617I}$ mouse model is not unexpected, as thrombocytosis is not commonly observed in *JAK2*$^{V617F}$ MPN mouse models. Therefore, we performed a more thorough assessment of the effect of *JAK2*$^{V617I}$ on hematopoietic progenitors and myeloid cells including megakaryocytes at time of sacrifice.

Mice were euthanized at 120 days post-transplant to fully assess the MPN phenotype. Spleen weight of *JAK2*$^{V617I}$ mice was not statistically different than empty vector mice (Figure 1D), however we found a positive correlation between spleen weight and percentage of GFP-positive cells in the spleen (Supplemental Figure 2). As expected, spleen weight was increased in *JAK2*$^{V617F}$ mice (Figure 1D). On histologic inspection *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ mice had hypercellular marrows with increased numbers of megakaryocytes. Mild fibrosis was seen in *JAK2*$^{V617I}$ mice, severe reticulin fibrosis was seen in *JAK2*$^{V617F}$ mice. The splenic architecture was preserved in *JAK2*$^{V617I}$ mice, whereas in *JAK2*$^{V617F}$ mice the splenic architecture was disrupted by invasion of myeloid cells including megakaryocytes (Figure 1E). These data demonstrate that ectopic expression of *JAK2*$^{V617I}$ in hematopoietic cells induces histologic evidence of MPN but with a milder phenotype as compared to *JAK2*$^{V617F}$.

### *JAK2*$^{V617I}$ expands myeloid progenitors and megakaryocytes and mobilizes myeloid progenitors to the spleen

To identify whether *JAK2*$^{V617I}$ affects the frequency of hematopoietic stem and progenitor cells or expands mature myeloid lineage cells we compared these populations in *JAK2*$^{V617F}$, *JAK2*$^{V617I}$, and MIG empty vector mice (Figure 2A–C). The bone marrow of *JAK2*$^{V617I}$ mice had expanded GMP and MEP populations as compared to MIG empty vector and *JAK2*$^{V617F}$ mice (Figure 2A). Mature granulocyte (Gr-1$^{+}$CD11b$^{+}$) and erythroid (Ter119$^{+}$) populations were expanded in *JAK2*$^{V617F}$ but not *JAK2*$^{V617I}$ mice. To determine whether *JAK2*$^{V617I}$ had mobilized myeloid progenitors to the spleen we compared myeloid colony formation ability from harvested spleen cells of MIG, *JAK2*$^{V617F}$, and *JAK2*$^{V617I}$ mice. We found increased myeloid progenitor activity in the spleens from *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ mice as compared to MIG empty vector (Figure 2D).

Both *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ mice had an increased fraction of megakaryocytes (CD41$^{+}$) in the spleen as compared to empty vector mice (Figure 2B). To evaluate megakaryopoiesis in more detail we sorted lineage$^{neg}$, c-kit$^{+}$, Sca-1$^{+}$ (LKS) cells ectopically expressing *JAK2*$^{V617I}$, *JAK2*$^{V617F}$ or MIG empty vector onto OP9 stromal cell layers (100 cells/well) in the presence of mouse thrombopoietin (mTPO) (10ng/ml), Stem Cell Factor (mSCF) (50ng/ml), and Interleukin-11 (mIL-11) (10ng/ml) to induce development of megakaryocytes. After 5 days in culture cells were harvested and analyzed by flow cytometry to identify megakaryocytes. Wells seeded with *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ cells had an increased percentage of megakaryocytes (CD41$^{+}$ CD42d$^{+}$) as compared to wells seeded with empty vector cells (Figure 2E). We also compared the ability of *JAK2*$^{V617I}$, *JAK2*$^{V617F}$, and empty vector hematopoietic progenitors to form megakaryocyte colonies in methylcellulose and found a skewing toward megakaryocyte colonies in both *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ as compared empty vector (Figure 2F). Together, these data demonstrate that expression of *JAK2*$^{V617I}$ drives the expansion of myeloid progenitors and megakaryocytes despite the lack of overt leukocytosis or thrombocytosis in peripheral blood.

### *JAK2*$^{V617I}$ results in cytokine hypersensitivity without constitutive activation

Humans with germline *JAK2*$^{V617I}$ mutations do not display constitutive activation of the kinase but they do demonstrate cytokine hyper-responsiveness as evidenced by increased phosphorylation of STATs at low concentrations of ligand[11]. We compared phosphorylated STAT5 in peripheral blood cells taken from *JAK2*$^{V617I}$, *JAK2*$^{V617F}$, and MIG empty vector mice following stimulation with increasing concentrations of GM-CSF. At all concentrations of GM-CSF tested *JAK2*$^{V617I}$ and *JAK2*$^{V617F}$ mice had exaggerated phosphorylation of STAT5 as compared to MIG empty vector mice (Figure 3A). We also measured phospho-STAT3 and STAT5 in unstimulated bone marrow and spleen from each mouse at time of sacrifice, there was no difference between *JAK2*$^{V617I}$ and MIG empty vector mice. *JAK2*$^{V617F}$ mice did demonstrate phosphorylation of STAT3 and STAT5 even in the absence of cytokine stimulation, confirming the ability of *JAK2*$^{V617F}$ but not *JAK2*$^{V617I}$ to constitutively activate downstream signaling pathways (Figure 3B).

The *JAK2*$^{V617I}$ mouse transduction-transplantation model has phenotypic features of acquired MPN such as expansion of megakaryocytes and mobilization of hematopoietic progenitors to the spleen but the phenotype is not as robust as *JAK2*$^{V617F}$. It is possible that all cells in the hematopoietic system must express *JAK2*$^{V617I}$, as is the case for a germline mutation, in order for *JAK2*$^{V617I}$ to make a clinically relevant impact in humans. Although the *JAK2*$^{V617F}$ mutation is somatically acquired in MPN familial clustering is well described[7,10]. Genome-wide analyses have revealed that JAK2$^{V617F}$-positive MPN is strongly associated with a specific constitutional JAK2 haplotype (designated 46/1)[17–19], suggesting that germline variation is an important contributor to MPN phenotype and predisposition.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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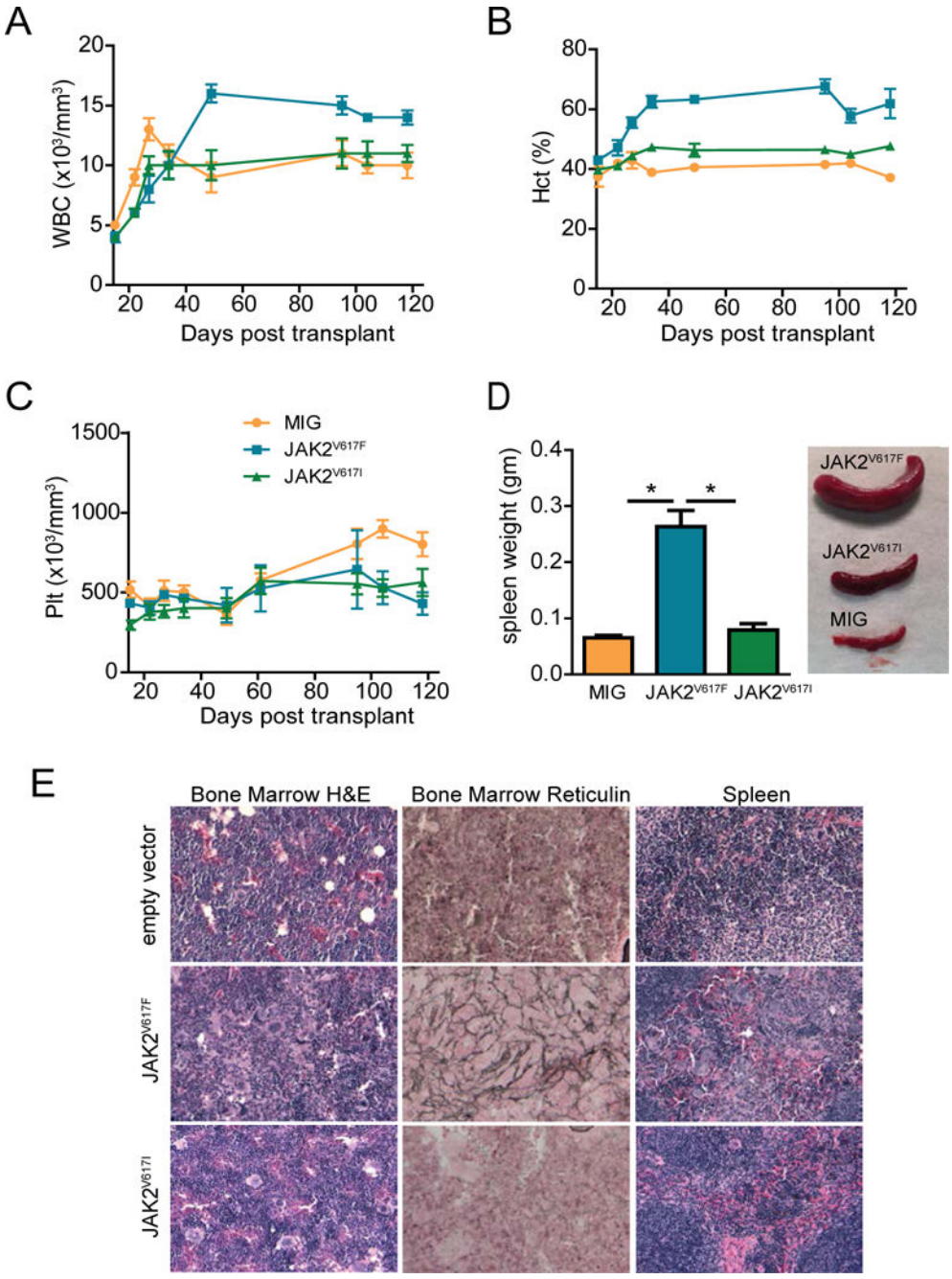


**Figure 1. *JAK2*$^{V617I}$ causes a mild MPN phenotype**

**A–C.** Peripheral blood was drawn weekly from MIG empty vector, *JAK2*$^{V617I}$, and *JAK2*$^{V617F}$ mice. WBC (A), HCT (B) and platelets (C) were measured using a VetABC hematology analyzer (scil). **D.** Spleen weight and a representative photo of MIG empty vector, *JAK2*$^{V617I}$, and *JAK2*$^{V617F}$ mice. **E.** Bone marrow and spleen from each mouse was evaluated by a blinded pathologist. Representative H&E sections of bone marrow, spleen and reticulin stain of bone marrow are shown (20X magnification shown, Leica DC300 camera running IM50 Image Manager software).

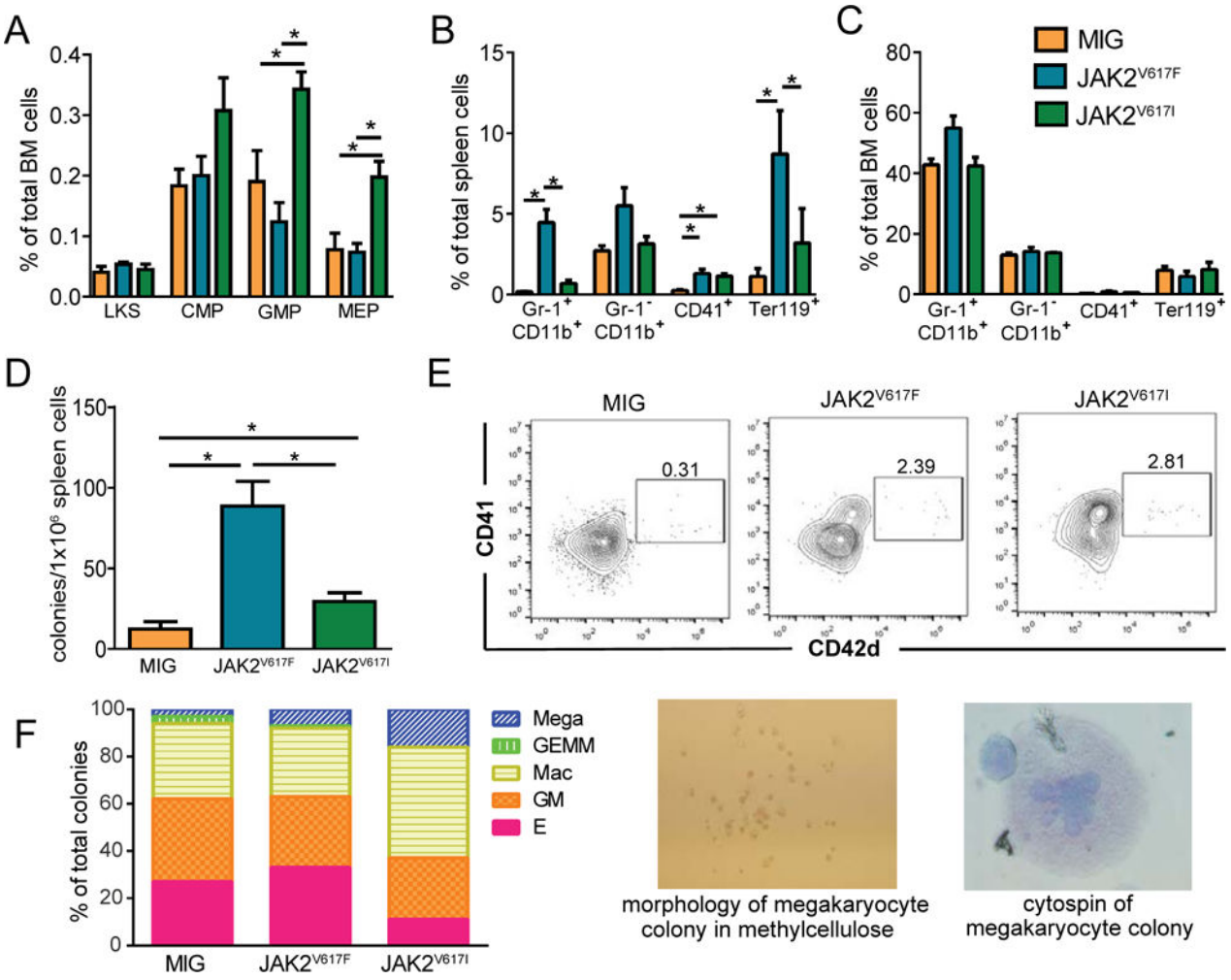


**Figure 2. *JAK2*$^{V617I}$ expands myeloid progenitors and megakaryocytes**

**A.** Frequency of hematopoietic stem (LKS), common myeloid progenitors (CMP), granulocyte monocyte progenitors (GMP) and megakaryocyte erythroid progenitors (MEP) in the bone marrow of each mouse measured by flow cytometry. **B,C**. Frequency of granulocytes (Gr-1$^+$ CD11b$^+$), monocytes (Gr-1$^-$ CD11b$^+$), megakaryocytic lineage (CD41$^+$) and erythroid lineage cells (Ter119$^+$) in spleen (B) and bone marrow (C) measured by flow cytometry. **D.** Myeloid colony formation (CFU-GM and CFU-E combined) in methycellulose (M3231, StemCell Technologies) supplemented with mSCF, mIL-3 (peprotech) and hEpo (Procrit, Amgen). **E.** In vitro differentiation of megakaryocytes (CD41$^+$ CD42d$^+$) from LKS cells on OP9 stromal cell layers (+mTPO, mIL-11, mSCF). F. Relative frequency of myeloid colony types from JAK2$^{V617F}$, JAK2$^{V617I}$, and empty vector hematopoietic progenitors. Progenitors were plated in methylcellulose (M3231, StemCell Technologies) supplemented with mSCF, mIL-3, hEpo, and mTPO. After 7 days colonies were scored morphologically and enumerated. Photos of a representative megakaryocyte colony are shown to the right. All bar graphs represent mean (+/-SEM), *denotes $p<0.05$.

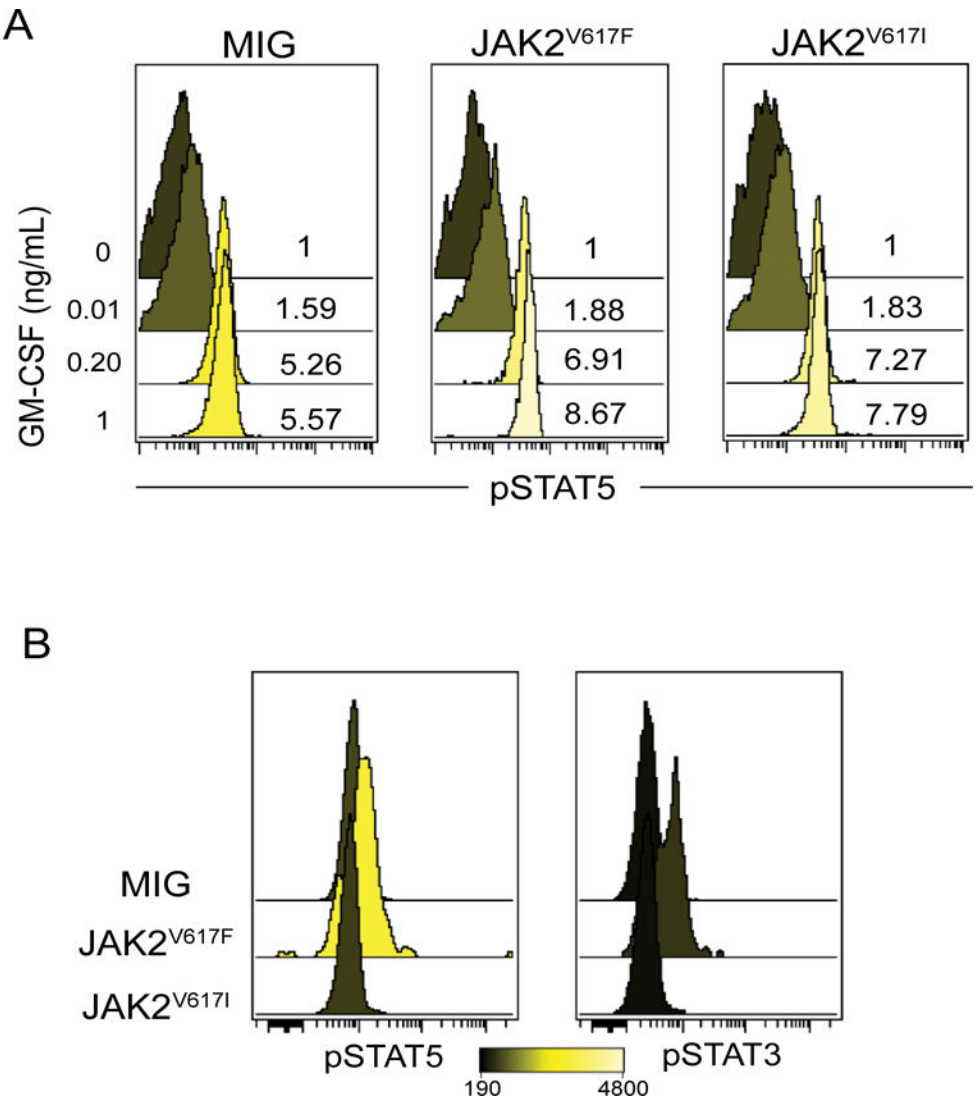


**Figure 3. *JAK2*$^{V617I}$ results in cytokine hypersensitivity without constitutive activation**

**A.** Peripheral blood from each group of mice was pooled and stimulated with increasing concentrations of mGM-CSF and then analyzed for phospho-STAT5 (pY694) by flow cytometry. Fold increase in phospho-STAT5 over unstimulated is shown as histogram overlay (gated on GFP$^+$ cells). **B.** Unstimulated bone marrow from MIG, JAK2$^{V617F}$ and JAK2$^{V617I}$ mice were analyzed for levels of phospho-STAT5 (pY694) and phospho-STAT3 (pY705) using flow cytometry, histogram overlay represents (MFI) of a representative mouse from each group (gated on GFP$^+$ cells).