UNIVERSITY OF CALIFORNIA,
IRVINE

The Role of IL-10R Signaling in the Production and Effects of Chronic Inflammation in Myeloproliferative Neoplasms (MPNs)

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

By

Hew Yeng Lai

Dissertation Committee:
Professor Angela Fleischman, Chair
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2021

DEDICATION

To

My doctoral advisor – Angela G. Fleischman

My mother, father, and brother

My lifelong friends

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ABSTRACT OF THE DISSERTATION

The Role of IL-10R Signaling in the Production and Effects of Chronic Inflammation in Myeloproliferative Neoplasms (MPNs)

By

Hew Yeng Lai

Doctor of Philosophy in Biological Chemistry

University of California, Irvine, 2021

Associate Professor Angela G. Fleischman, Chair

Myeloproliferative Neoplasm (MPNs) are a group of chronic blood cancers characterized by the expansion of a mutant clone with constitutive activation of JAK/STAT signaling, resulting in an overproduction of myeloid cells. MPN patients have an increased risk of thrombosis and transformation to acute leukemia. There is no cure for MPN besides bone marrow transplantation, and current treatments only alleviate symptom burden and reduce thrombotic risk. Of the stem cell derived mutations identified in MPN, *JAK2*^{V617F} is the most common acquired driver mutation in hematopoietic stem cell (HSC).

Interestingly, several groups including our lab have demonstrated that the *Jak2*^{V617F} HSCs do not have a selective advantage over wild-type HSC. In order for a JAK2^{V617F} HSC to expand and cause disease, it must have gained a selective advantage to outcompete normal HSC. This suggests that specific environmental pressures must be present for *JAK2*^{V617F} mutant cells to gain a selective advantage, outcompete their normal counterparts and expand to cause an MPN.

Chronic inflammation is a key feature of MPN. Inflammation not only drives many of the debilitating symptoms associated with MPN such as severe itching and fatigue, but also

provides an environment that is advantageous to MPN mutant HSC. The mechanism driving chronic inflammation in MPN is currently unknown. Therefore, by understanding the underlying cause of inflammation that drives the pathogenesis of MPN, we could provide better therapeutic targets and prevention for disease development.

This work addresses what causes exaggerated inflammatory cytokine productions and its role in clonal expansion of mutant HSC in MPN patients. We have found that monocytes from MPN patients have impaired IL-10R signaling, which normally serves to reduce inflammation. This results in the inability of MPN monocytes to dampen pro-inflammatory cytokine, TNF-α. We have also found that the overproduction of TNF-α and IL-10R signaling defect are independent of the *Jak2*^{V617F} mutant cells, suggesting that there may be a genetic feature of those predisposed to acquire MPN. Using a *Jak2*^{V617F} knock-in mouse model, where the *Jak2*^{V617F} mutant HSCs do not normally have a selective advantage over WT HSCs, we have found that blocking IL-10R provide a selective advantage for clonal expansion. Collectively, these studies suggest that the IL-10R signaling defects in MPN patients plays an important role for clonal expansion and disease development.

CHAPTER 1

INTRODUCTION

1.1 Background on Myeloproliferative Neoplasms (MPNs)

Myeloproliferative Neoplasm (MPNs) are a group of chronic hematological malignancies characterized by the excessive production of terminally differentiated myeloid cells [1]. MPNs are categorized into three subgroups based on the diagnostic criteria: Polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) [2]. Patients with PV has elevated red blood cell mass and megakaryocytic/granulocytic hyperplasia. Patients with ET have increased platelet productions and hyperplasia of the megakaryocytic and granulocytic linages. Patients with MF has bone marrow failure due to the buildup of collagen fibers that disrupt the normal blood production of the hematopoietic compartment, which leads to immature hematopoietic cells expanding to the spleen, resulting in an enlarged spleen [3, 4]. All three subgroups of MPN feature bone marrow hypercellularity, increase risk of thrombosis and transformation to acute myeloid leukemia, which is a more fatal progression in blood cancer.

MPNs arise from a single somatic mutation in a hematopoietic stem cell (HSC) that clonally expand to give rise to myeloid lineage cells [5, 6]. The primary MPN driver mutations, including those in Janus Kinase 2 (*JAK2)*, Calreticulin (*CALR*), and Myeloproliferative Leukemia Virus (*MPL*), which abnormally activate the cytokine receptor JAK2/STAT pathway [7, 8]. These driver mutations in MPN are mostly mutually exclusive and are used in prognosis. The most prevalent somatic driver mutation is the substitution of valine to phenylalanine at codon 617 of JAK2 (*JAK2*^{V617F}), which is an activating mutation caused by a point mutation G to T at nucleotide 1849, in exon 14 [9, 10]. The *JAK2*^{V617F} mutation accounts for about 95% of PV, and 50-60% of ET and MF. CALR and MPL mutations are absent in PV and account for 20-25% and 3-

4% in ET and 20-25% and 6-7% in MF, respectively. About 10-15% of MPN patients do not have any of these three mutations and are referred to as triple negative [2].

JAK2 is a non-receptor tyrosine kinase that is required for signal transduction for many type I cytokine receptors such as MPL, erythropoietin (TPO) receptor, and granulocyte colony stimulating factor (GCSF) receptor [11]. They are essential receptors in the development and proliferation of myeloid lineage cells. The V617F mutation occurs in the pseudokinase domain (JH2) in JAK2, and this results in constitutive activation of the JH1 kinase domain (figure 1.1). The mutation in the JH2 domain disrupt it's autoinhibitory function, which leads to JAK2 signaling in the absence of appropriate ligands ligation to the cytokine receptor (figure 1.2) [5, 10]. This leads to cytokine independent expansion of mature myeloid cells.

MPN symptom burdens has significant impact on the quality of life. Common symptoms in MPN include fatigue, abdominal pain, headache, insomnia, numbness, nights sweats, fever, and weigh loss [12, 13]. Current drug treatment for MPN, including aspirin, busulfan, 6-mercaptopurin, and hydroxyurea, can be helpful in alleviating symptoms, but the responses are of short duration and patients have develop resistances to these medications [14]. Ruxolitinib, a JAK 1/2 inhibitor, is an FDA approved treatment for MF patients with enlarged spleen, has shown to have the ability to reduce splenomegaly and suppress the production of inflammatory cytokine in MPN patients [15]. However, Ruxolitinib has not shown to be curative for MPN, and can cause various side effects in a long-term treatment. Although there are many JAK inhibitors that were being research for clinical trials, it is now recognized that targeting JAK2 alone is not sufficient [16, 17]. Allogeneic hematopoietic cell transplantation is the only potential curative therapy, which is rarely performed due to high risk of treatment-related mortality. Therefore,

there is an ongoing need for the development of therapeutic treatment that can modify the natural history of MPN.

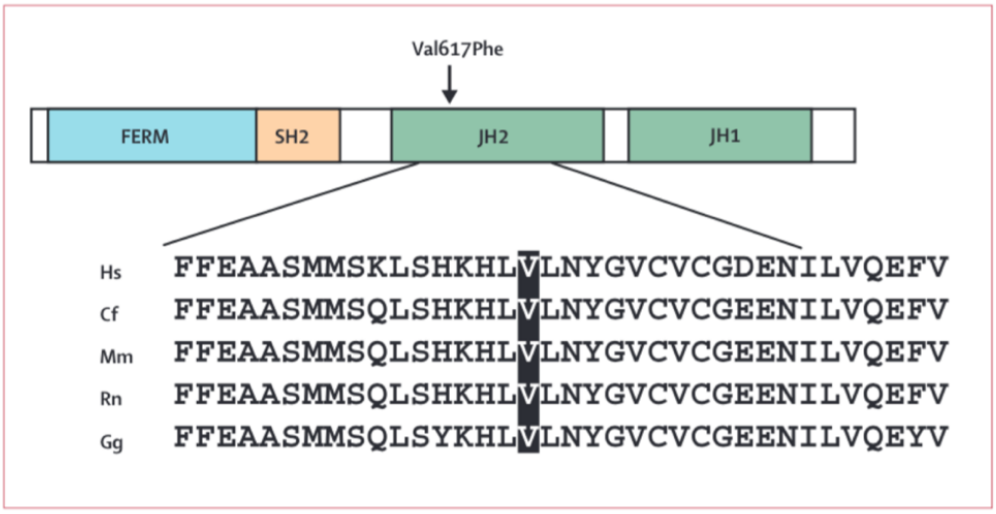

Figure 2: Diagrammatic representation of JAK2
FERM, SH2, JH2, and JH1 domains are shown, together with a sequence alignment of part of the JH2 domain.
Hs=*Homo sapiens*. Cf=*Canis familiaris*. Mm=*Mus musculus*. Rn=*Rattus norvegicus*. Gg=*Gallus gallus*.

Figure 1.1 Diagrammatic representation of JAK2. FERM, SH2, JH2 and JH 1 domains are shown, together with a sequence alignment of JH2 domain. HS= Homo sapiens. CF= Canis familiaris. Mm= Mus musculus. Rn= Rattus norvegicus. Image citation [5]

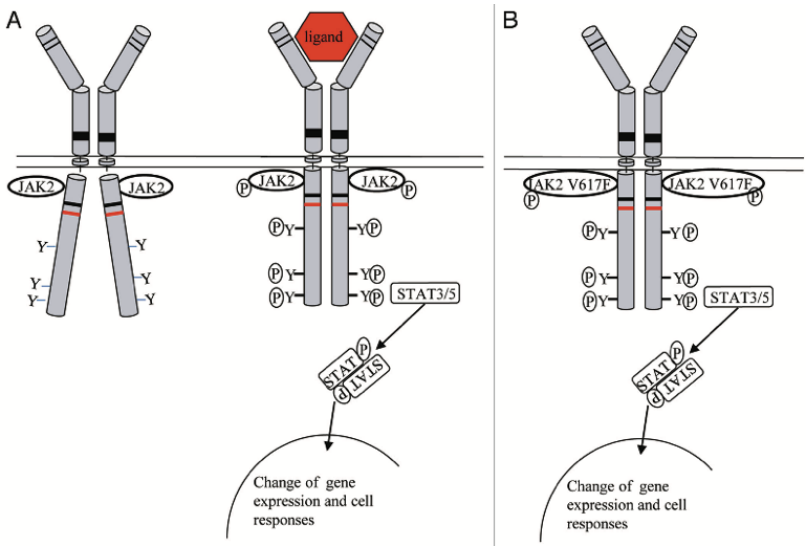

Figure 1.2 JAK2 signaling and the effect *JAK2*^{V617F} on JAK-STAT signaling activation. Image citation [18]

1.2 The Impact of Chronic Inflammation on MPN

Myeloproliferative Neoplasm (MPN) is a type of blood cancer that is associated with high inflammatory burden. MPN patients were shown to have high levels of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ), interleukin (IL)-6, IL-8, and IL-12 in the peripheral blood (figure 1.3). Chronic inflammation is responsible for many of the debilitating symptoms in MPNs [19]. For example, activation of megakaryocytes for platelet productions by inflammatory cytokine contribute to high risk of thrombosis. Emerging studies have suggested that inflammation is an important facilitator of disease development and progression, but exactly how inflammation mediates MPN initiation remains unclear [20, 21].

Inflammation is a host response to infection and or tissue injury to heal the wound. Leukocytes become activated and migrate to the site of damage and secrete small proteins called inflammatory cytokines, including chemokine, interleukin, lymphokine and monokines [22]. Pro-inflammatory cytokines are produced predominately by activated macrophages upon triggered by receptors in the innate immune system such as Toll-like receptors (TLRs) [23]. This process is important for activation of effector leukocytes to kill invading pathogens, such as activating neutrophils to release toxic granules inside the cells, including reactive oxygen species (ROS) and reactive nitrogen species. Acute inflammatory response is essential in eliminating disease causing pathogens and proceed by a tissue repair phage by tissue-resident macrophages. Anti-inflammatory cytokines are immunoregulatory protein is essential in modulating the inflammatory response. Major anti-inflammatory cytokines include IL-4, IL-10, IL-11, and IL-13, are important in restoration of homeostasis. In particular, IL-10 has potent

anti-inflammatory activities to dampen the expression of TNF-α, IL-1, and IL-6 in activated macrophages [22]. Chronic inflammation occurs when stimulation of inflammatory cytokine persists by activating immune cells, causing tissue damages and leads to autoimmune responses [24].

Many studies have reported that MPN is associated with a variety of autoimmune diseases including, Crohn's disease, rheumatoid arthritis, immune thrombocytopenic purpura, and progressive systemic sclerosis [25, 26]. In a large study conducted in a Swedish epidemiological group, it was found that individuals with a history of any autoimmune disease were associate with a significantly high risk of developing MPN [27]. In a separated Danish study, it was found that MPN patients were 40% more likely to be diagnosed previously to have an inflammatory bowel disease (IBD) [28]. Interestingly, the frequency of 46/1 haplotype, which is a predisposition to acquiring MPN, is found to be increased in Crohn's disease [29, 30]. Although the link between chronic inflammation and MPN has been firmly established, it is remains unclear whether the aberrant inflammation occurs before or after the development of MPN.

Chronic inflammation has long been found to have a strong association with the development of cancer [31, 32]. Many cancers occur at the site of infection or inflammation because an inflamed microenvironment drive oncogenesis (figure 1.4). Cancerous cells have also been shown to have resistance in such microenvironment, which is hypothesized to give them an advantage to expand clonally over wild-type cells [31]. TNF-α is an aging-associated pro-inflammatory cytokine that is elevated in MPN patients [33]. The Fleischman lab has

demonstrated that the *JAK2*^{V617F} mutant hematopoietic progenitors are resistant to the negative effects of TNF-α compared to their WT counterparts (figure 1.5) [34].

The mechanism driving chronic inflammation in MPN and defining how JAK2^{V617F} mutant and wild-type HSC respond differently is critical to understand how JAK2^{V617F} mutant HSC gain a selective advantage and initiate disease. Identifying the mechanism driving chronic inflammation in MPN and defining how JAK2^{V617F} mutant and wild-type HSC respond differently this inflammation is critical to understand how JAK2^{V617F} mutant HSC gain a selective advantage and initiate disease.

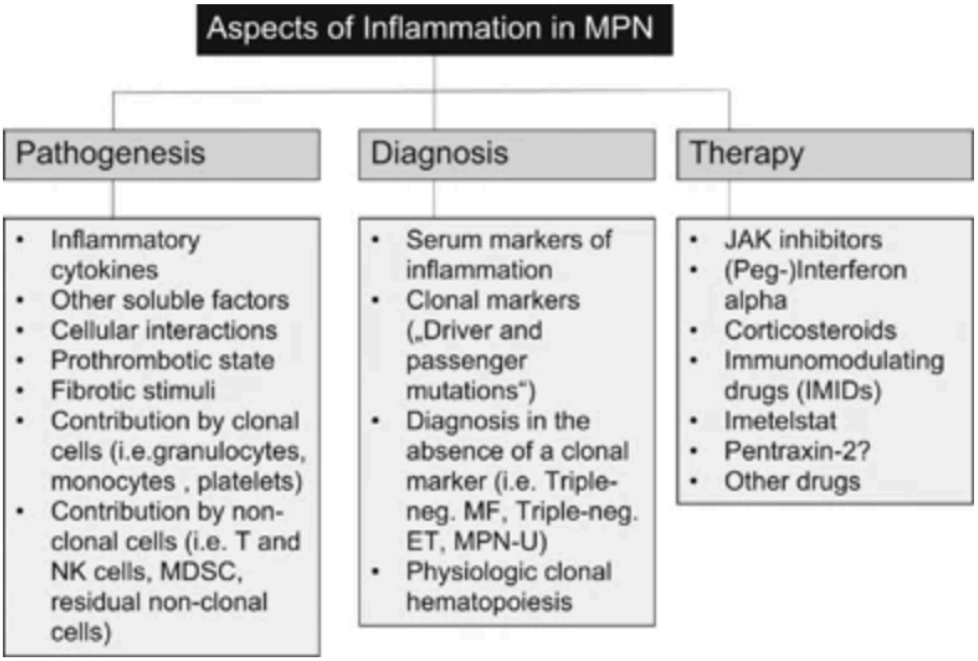

Figure 1.3 Chronic inflammation in MPN drives many of the pathogenesis of the disease. Inflammatory cytokines can be used as diagnosis markers. Image citation [35]

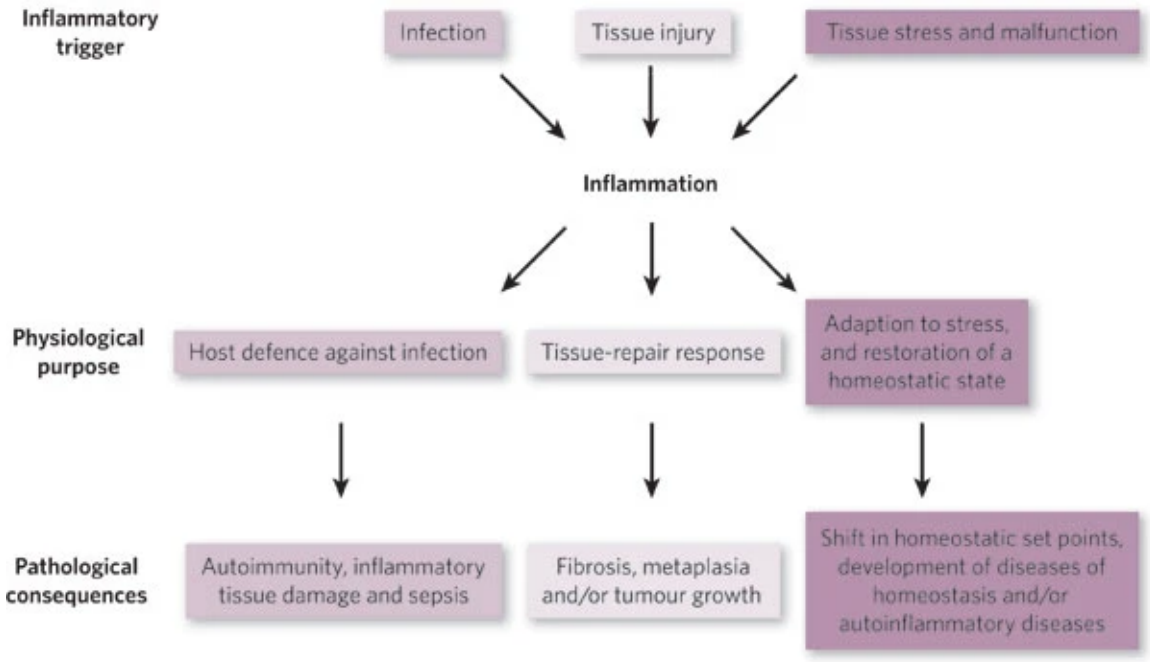

Figure 1.4 Chronic Inflammation is known to be responsible for numerous physiological responses in MPN disease progression. Image citation [23]

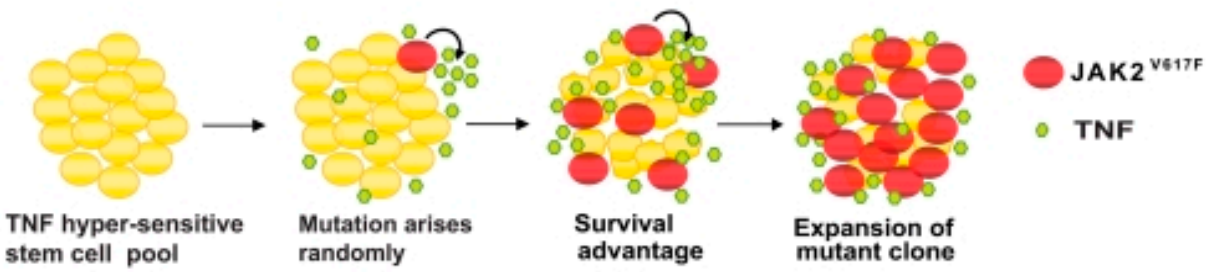

Figure 1.5 Summary Model of TNF-α facilitates clonal expansion in MPN. Image citation [34]

1.3 Clonal Hematopoiesis of MPN

Clonal hematopoiesis is defined as the expansion of a clonal blood cell population with a somatic mutation, which is essential in developing hematological malignancy [36]. Hematopoietic stem cells (HSCs) are blood stem cells that have the unique ability to self-renewal and differentiate into multipotent progenitor (MPPs), which become committed to

differentiating into common lymphoid progenitor (CLPs) or common myeloid progenitors (CMPs) to produce all blood cell types [37]. Somatic mutations in HSCs arise from the onset of development stage and accumulate as we age (figure 1.6). Not all mutations lead to clonal expansion because only those that allow cells to grow at a faster rate than their counterpart have an advantage. This process of clonal selection often requires a change in the microenvironment to give the clone a selective advantage, referred to as the evolution of clonal selection in cancer [38-40]. This clonal selection and expansion of a mutant cell strongly depends on the interaction between the clone and its tissue habitats.

MPN is a stem cell derived clonal disorder arises from a somatic mutation that leads to constitutive activation of thrombopoietin receptor signaling, most commonly a *JAK2*^{V617} mutation, in HSC. For a JAK2^{V617F} HSC to expand and cause disease, it must have gained a selective advantage to outcompete normal HSC (figure 1.7). The variant allele frequency in MPN *JAK2*^{V617} has been detected at less than 1% of VAF in the normal population [41]. Although many studies have identified the acquisition of germline variants such as TET2, TERT, ATM and the haplotype (GGCC) referred to as 46/1 in the JAK2 gene having a higher risk of developing MPN[29, 42], the predisposition to acquiring *JAK2*^{V617} remains unclear.

Exposure to chronic inflammation has been shown to have key contribution to selection of malignant HSC clones [43]. HSCs are a rare population that are largely in quiescent in order to preserve their functional property. Upon specific stress such as TLR activation and response to inflammatory cytokines, HSCs are activated and are triggered to divide and produce mature leukocytes, preferentially differentiate toward the myeloid lineages, to counter infections [44,

45]. Therefore, chronic inflammation has significant effects on HSCs exhaustion and may contribute to the development of blood cancer.

Many studies have indicated that *JAK2*^{V617} HSCs do not confer a self-renewal advantage and do not demonstrate an advantage in competitive transplantation experiments [46, 47]. The acquisition of the *JAK2*^{V617} mutation was found to be a late event, which suggest that other genetic events or factors are responsible for clonality [48]. Therefore, *JAK2*^{V617} alone is insufficient to initiate disease and that additional factors are required. In addition, there are several evidence to show that relatives of MPN patients have about five to seven fold increase risk of developing MPN, which are termed familial MPN [49, 50]. In some MPN families, all affected members acquire the *JAK2* V617F mutation, while others harbor different mutations including *JAK2* V617F, *MPL*, and *CALR* (figure 1.8) *[50-52]*. We reason that a specific stressor must be presented to reveal the selective advantage of *JAK2*^{V617F} mutant cells. This stressor may be presented in MPN families, and this could explain why clones with MPN driver mutations such as *JAK2*^{V617F} independently emerge in multiple family members [53]. A goal of my project is to determine whether chronic inflammation is a predisposition of MPN. This will help us understand what the specific stressors are in driving clonal expansion in this disease.

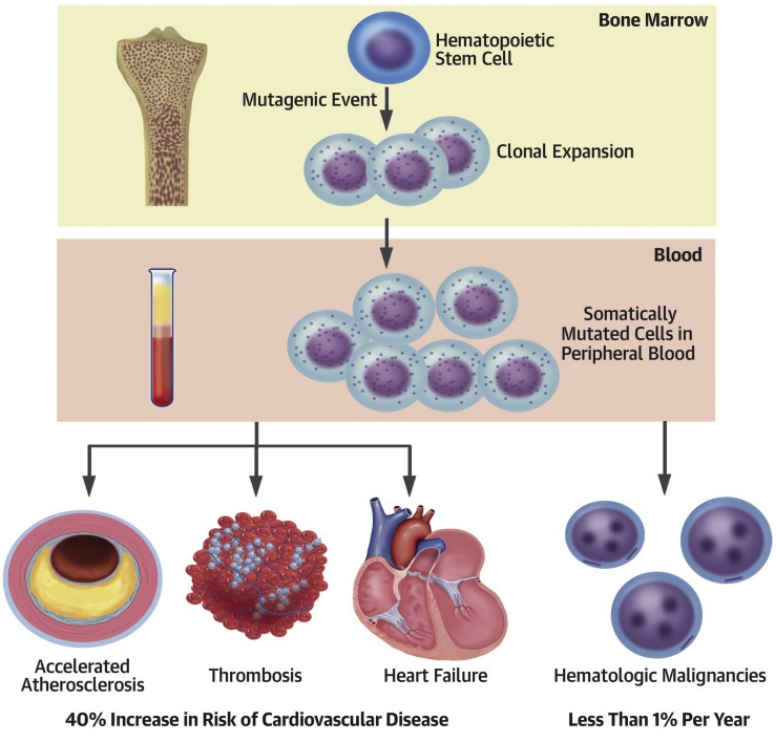

Figure 1.6 The development of malignant clone in hematopoietic stem cells and clonal expansion that drives pathogenesis. Image citation [55]

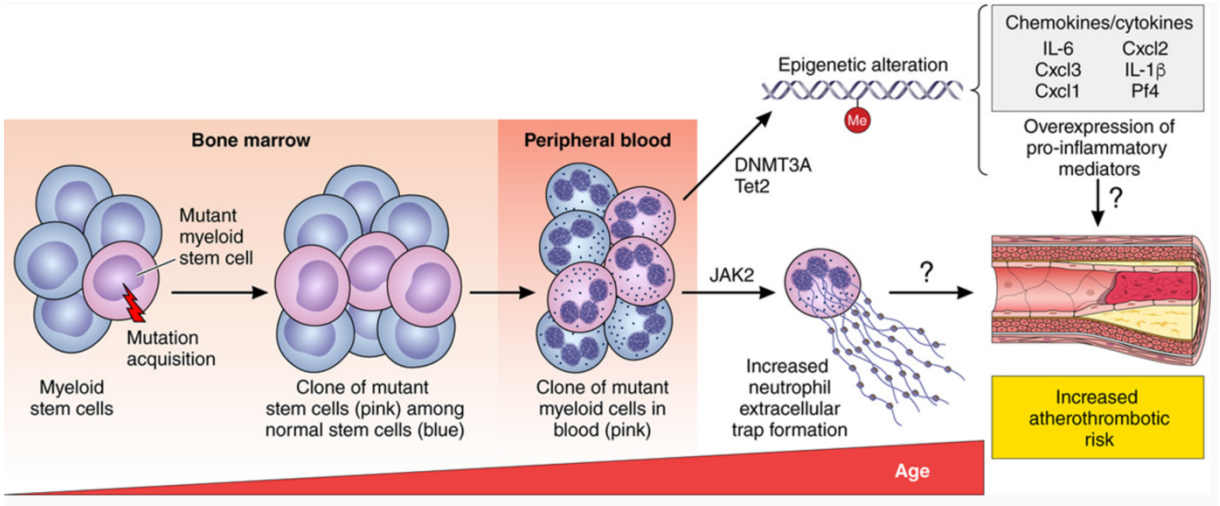

Figure 1.6 Model of Clonal Hematopoiesis in MPN. Image citation [54]

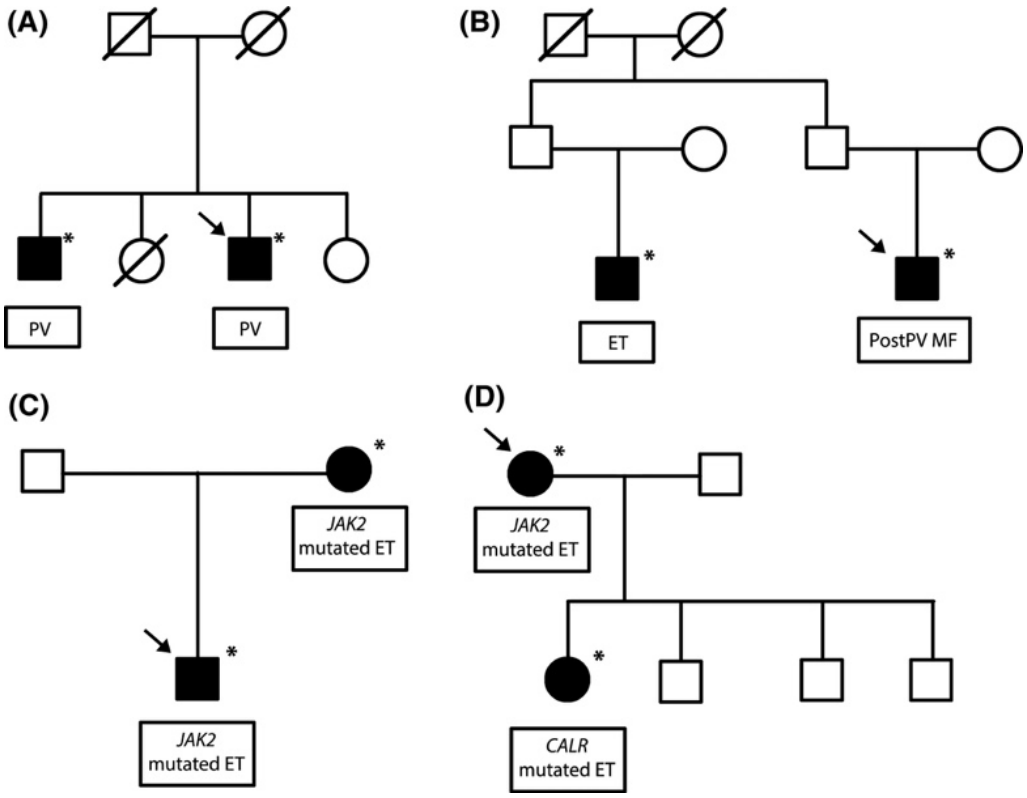

Figure 1.8 Representative pedigrees of familial myeloproliferative neoplasms. (A) pedigree with homogenous clinical phenotype: all affected relatives with PV. (B) pedigree with heterogenous clinical phenotype: one relative affected with ET, one relative affected with post-PV myelofibrosis. (C) pedigree with homogeneous molecular status: all affected relatives carry *JAK2* V617F mutation. (D) pedigree with heterogeneous molecular status: one relative affected with *JAK2*-mutated ET, one relative affected with *CALR*-mutated ET. Arrows indicate the probands, asterisks indicate patients with DNA available for molecular studies.
Image citation [50]

1.4 The Role of IL-10R signaling in Inflammation and Hematopoietic Stem Cells

Inflammation has a significant impact in the pathogenesis of MPN in both driving its symptoms and the clonal expansion of mutant cells. To understand the origin of the defects, it is important to examine the key pathway in inflammatory modulation. IL-10 is a class 2 cytokine that potently inhibits the production of pro-inflammatory cytokines. The respond to IL-10 cytokine requires the surface receptors of IL-10R1 and IL-10R2 chains (figure 1.9)[56]. IL-10R1 has a specific high-affinity interaction with IL-10, whereas IL-10R2 is low affinity can be bind to

other class 2 cytokine family members such as IL-20, IL-22, and IL-28 [57, 58]. IL-10 binding to the receptor complex activates JAK1 and TYK2, which phosphorate IL-10R1 to activate transcription factor STAT1, STAT3, and STAT5 [59-61]. Thus, the IL-10R signaling cascade is more specific to IL-10R1 than IL10R2.

The immunosuppressive function of IL10 is well-known in both human and animal models [62]. Many studies have found that the impairment of the IL-10 signaling pathway leads to inflammatory diseases. Mutation in IL10, IL10R1 and IL-10R2, which disrupt the IL-10 signaling, were found to cause an early onset of inflammatory bowel disease (IBD) [63, 64]. Consistently, mice with IL-10 knockout (KO) develop severe IBD [65]. Several studies have also identified the strong association of IL-10 defects with other autoimmune diseases, including systemic lupus erythematosus, Crohn's disease, encephalomyelitis, and ulcerative colitis [66-69]. Because autoimmune diseases were found to have a significant increased risk of developing MPN as previously mentioned (in section 1.2), it is of our interest to understand whether IL-10 signaling has a role in the pathogenesis of MPN.

In addition to having an essential role in regulating inflammation in many mature immune cells, IL-10 is also recently found to maintain the function of hematopoiesis in HSCs [70-73]. It was found that loss of IL-10 results in reduced HSC population and negatively impact their self-renewal function [74]. This suggest that IL-10 signaling promotes self-renewal in HSCs and protect them from excessive proliferation. Although majority of HSCs are typically in quiescent, they can be induced to proliferation and differentiation in response to stress signal, such as TLR ligation. Chronic stress such as inflammation was found to lead to accelerations of HSC exhaustion and loss of this population [75]. Therefore, defects in the IL-10 signaling

pathway may have direct impact on reducing the fitness of HSCs. A major goal of this project was to understand how defects in the IL-10 signaling pathway impact WT HSCs compare to *JAK2^{V617} HSCs.* Elucidating this effect would allow us to determine whether IL-10 signaling impairment have a role in driving clonal proliferation in MPN.

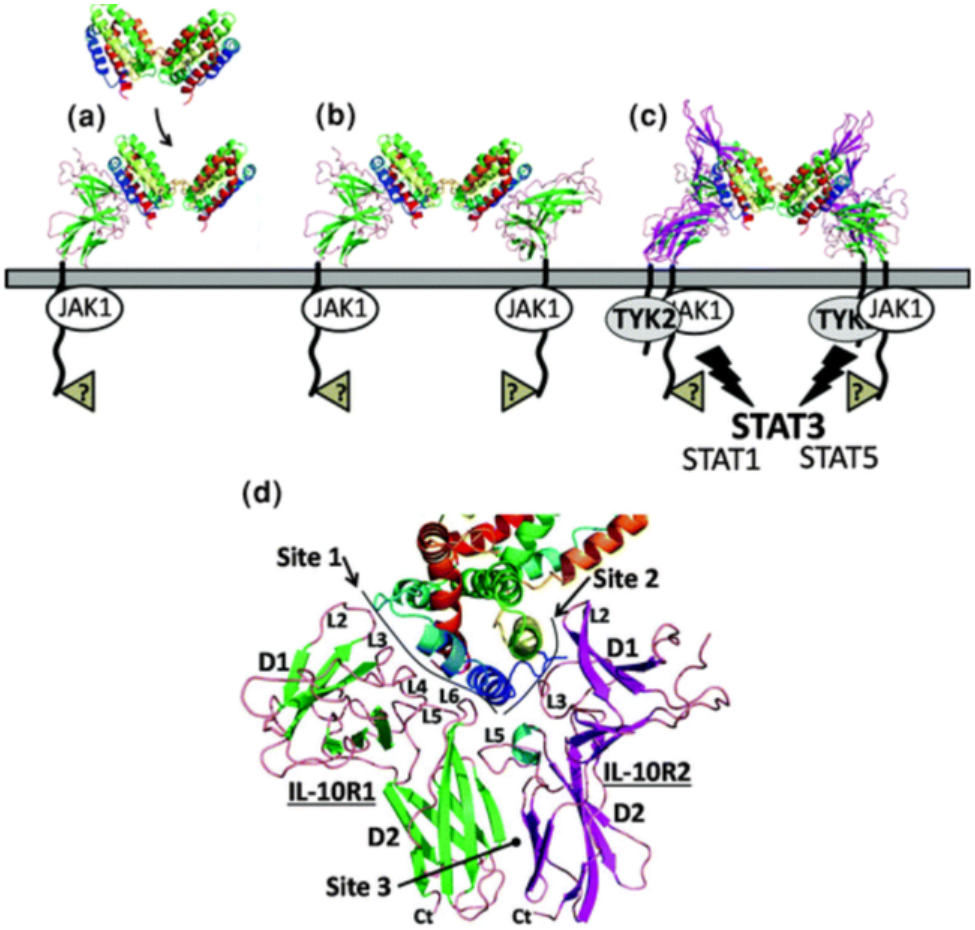

Figure 1.9 IL-10 ligand and receptor interaction in the IL-10 signaling pathway. Image citation [76]

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CHAPTER 2

Defective IL-10R Signaling Contributes to Excessive TNF-α Production in MPN Patients

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2.1 Introduction

Myeloproliferative neoplasm (MPN) is a chronic hematologic malignancy resulting from the somatic acquisition of a mutation which leads to constitutive activation of thrombopoietin receptor (MPL) signaling (*JAK2*^{V617F}, *CALR*, *MPL*) and subsequent expansion of mature myeloid cells. MPN patients have elevated serum inflammatory cytokine concentrations [1-3], and this chronic inflammatory state is responsible for the debilitating constitutional symptoms characteristic of this disease [4, 5]. Treatment with the JAK1/2 inhibitor ruxolitinib, the only FDA approved drug for MPN, reduces inflammatory cytokines coincident with improvement of constitutional symptoms [6, 7]. Inflammation is also critical for MPN progression. For example, we have previously identified a central role for the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in the clonal expansion of the JAK2^{V617F} mutant clone [8]. JAK2^{V617F} endows upon hematopoietic progenitors TNF-α resistance, giving the JAK2^{V617F} clone a selective advantage over their TNF-α sensitive JAK2^{WT} counterparts in high TNF-α environments. Therefore, targeting excessive TNF-α production should be therapeutically beneficial in MPN and understanding the mechanism that drives excessive TNF-α production in MPN will guide strategies to target TNF-α in this disease.

TNF-α is classically produced by monocytes following stimulation of toll-like receptors (TLR), crucial pattern recognition receptors for microbial products [9]. A tightly regulated negative feedback TLR signaling [10, 11], orchestrated by the anti-inflammatory cytokine interleukin-10 (IL-10) [12-14], is critical to avoid persistent production of inflammatory cytokines following TLR stimulation. Because of its integral role in inflammation and TNF-α production, we hypothesized that exaggerated TLR signaling contributes to the increased TNF-α

seen in MPN. This study identifies the signaling defects in the IL-10 signaling pathway that results in persistent TNF-α production in MPN monocytes.

2.2 Materials and Methods

Patients

Peripheral blood was obtained from patients with polycythemia vera (PV), essential thromobocythemia (ET), myelofibrosis (MF), MPN family members, or normal volunteers. All participants gave their informed consent for the studies conducted in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Boards of the University of California, Irvine, Portland Veteran's Affairs Medical Center, and the Oregon Health and Science University.

$CD14^+$ Monocyte Isolation

Peripheral blood mononuclear cells (MNCs) were isolated by density gradient using Ficoll-paque PLUS (GE Healthcare) and red blood cells were lysed by ammonium chloride potassium (ACK) lysing buffer. Monocytes were selected using human CD14 MicroBeads (Miltenyi Biotec) and MACS separation column per the manufacturer's instructions. Cells were cultured in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine (PSL) and 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2 humidified incubator.

TNF-α and IL-10 ELISA

Human $CD14^+$ monocytes or murine BMDMs were stimulated with LPS (Invivogen), R848 (Invivogen) or LPS and recombinant human or mouse IL-10 (PeproTech) for the indicated time. For studies measuring the impact of IL-10 or IL-10R blocking antibody on LPS induced TNF-α production, human recombinant IL-10 (Peprotech) or hIL-10R blocking antibody (BioLegend) were added to cells simultaneously with LPS. Supernatants were collected and centrifuged to remove cellular debris. Samples were flash-frozen and stored at $^-$80°C until quantification. TNF-α and IL-10 were measured using human or mouse Ready-SET-Go! ELISA kits (eBioscience) according to the manufacturer's protocol.

Measurement of intracellular TNF-α

Human $CD14^+$ monocytes or murine BMDMs were stimulated with LPS for the indicated time and treated with brefeldin A (BD Biosciences) for the final 4 hours of stimulation. Cells were collected and fixed with 2% paraformaldehyde followed by permeabilization with 0.005% saponin in phosphoflow staining buffer (PFSB: phosphate-buffered saline + 0.5% bovine serum albumin). Human $CD14^+$ monocytes were stained with PE-conjugated TNF-α antibody (BD Biosciences) and FITC-conjugated CD14 antibody (BioLegend). Murine BMDMs were stained with PE-conjugated TNF-α antibody (eBioscience) and PerCP/Cy5.5 F4/80 antibody (BioLegend). Cells were analyzed on a BD Accuri C6 flow cytometer.

Phosphoflow

Fresh whole peripheral blood was stimulated with LPS, R848, or recombinant human IL-10 for 15 minutes or 2 hours then fixed in 1.6% paraformaldehyde and permeabilized with methanol. R848- and LPS-stimulated cells were stained with APC CD33 (BioLegend) and FITC CD14 (BD Biosciences) to identify monocytes along with PE-conjugated phospho-p38 (pT180/pY182). IL-10 stimulated cells were stained with APC CD33 (BioLegend) and FITC CD14 (BD Biosciences) to identify monocytes along with PE-conjugated pSTAT3 (pY705) antibody. All phosphoflow antibodies purchased from BD Biosciences. Cells were analyzed on a BD Accuri C6 flow cytometer.

SOCS3 expression

The expression of Suppressor of Cytokine Signaling 3 (SOCS3) which is induced in response to IL-10 stimulation was quantified in $CD14^+$ monocytes. The monocytes were stimulated with recombinant human IL-10 (PeproTech) for 15 minutes, 1 hour, or 2 hours at 1, 5, 10, or 50ng/ml. Cells were pelleted and washed with PBS prior to lysis in TriPure reagent (Roche). RNA was extracted according to the manufacturer's instructions and reverse-transcribed using the SuperScript VILO cDNA synthesis kit (Life Technologies). PCR was performed on a LightCycler 480 instrument (Roche) using Maxima SYBR Green qPCR Master Mix (Thermo Fisher). SOCS3 primers were as follows: forward: 5'-CACTCTTCAGCATCTCTGTCG-3'; reverse: 5'-TCTCATTAGTTCAGCATTCCCG-3'. β-actin primers were as follows: forward: 5'-CATTGCCCGACAGGATGCAG-3'; reverse: 5'-CTCGTCATACTCCTGCTTGCTG-3'. Data were normalized to β-actin and analyzed via the Pfaffl method.

Statistical analysis

Data are presented as the mean ± SEM. Data were analyzed using Student's t-tests, one-way ANOVA with Sidak's post-hoc analysis, two-way ANOVA with Sidak's post-hoc analysis, or two-way ANOVA with Tukey's post-hoc analysis where appropriate (GraphPad Prism).

2.3 Results

MPN patients produce more TNF-α in response to the TLR agonists

We measured TNF-α production by $CD14^+$ monocytes stimulated with the TLR agonists LPS (TLR4) and R848 (TLR7/8), comparing MPN patients and normal controls (figure 2.1A). $CD14^+$ cells were purified from fresh peripheral blood and incubated for 24 hours in the presence of increasing concentrations of TLR agonists. The concentration of TNF-α in the supernatant of MPN patient monocytes was higher than that of normal controls in response to both LPS and R848 ($p \leq 0.05$).

Next, we compared the fraction of $CD14^+$ monocytes that were actively producing TNF-α immediately after TLR ligation in MPN patients versus normal controls. We stimulated MPN and normal control peripheral blood mononuclear cells with the LPS or R848 for four hours along with brefeldin A (BFA) to retain TNF-α inside the cell and then used intracellular flow cytometry to quantify the percentage of $CD14^+$ monocytes that were TNF-α^+ (figure 2.1B). In unstimulated and R848-stimulated cells, there was no difference in the percentage of TNF-α^+ $CD14^+$ monocytes in MPN and normal controls ($p > 0.05$). Surprisingly, normal controls had a higher fraction of TNF-α^+ cells than MPN patients following LPS stimulation ($p = 0.0001$). We also

measured TNF-α production using ELISA at early time points (4hrs) following LPS stimulation by and found no difference in the amount of TNF-α produced by MPN versus normal controls (Supplemental figure 2.1). Furthermore, MPN patients and normal controls have a similar fraction of $CD14^{+}CD16^{+}$ proinflammatory monocytes (Supplemental figure 2.2). Thus, the increased TNF-α production in response to TLR ligation cannot be explained by an increased fraction of monocytes actively producing TNF-α immediately after stimulation but instead may be due to persistent TNF-α production at later time points post stimulation.

Sustained activation of TLR signaling pathway in MPN monocytes

The mitogen activated protein kinase (MAPK) pathways are key signaling intermediates in the cellular response to TLR stimulation. Activation of p38 MAPK is necessary for production of TNF-α following LPS stimulation [15, 16]. Therefore, we next quantified induction of phosphorylated p38 MAPK in $CD14^{+}$ monocytes from MPN versus normal controls 15 minutes and 2 hours after stimulation with LPS and R848 using phosphoflow (figure 2.1C). At 15 minutes both LPS and R848 induced an equivalent fold induction of phospho-p38 in MPN and normal controls, demonstrating that initial signaling following TLR stimulation is not exaggerated in MPN. At two hours after stimulation with LPS and R848, however, phosphorylation of p38 was maintained or even increased in MPN patients, whereas at two hours after stimulation phosphorylation of p38 returned to baseline in normal controls. These data suggest that failure to dampen TLR signaling may be responsible for the persistent TNF-α production following TLR stimulation in MPN.

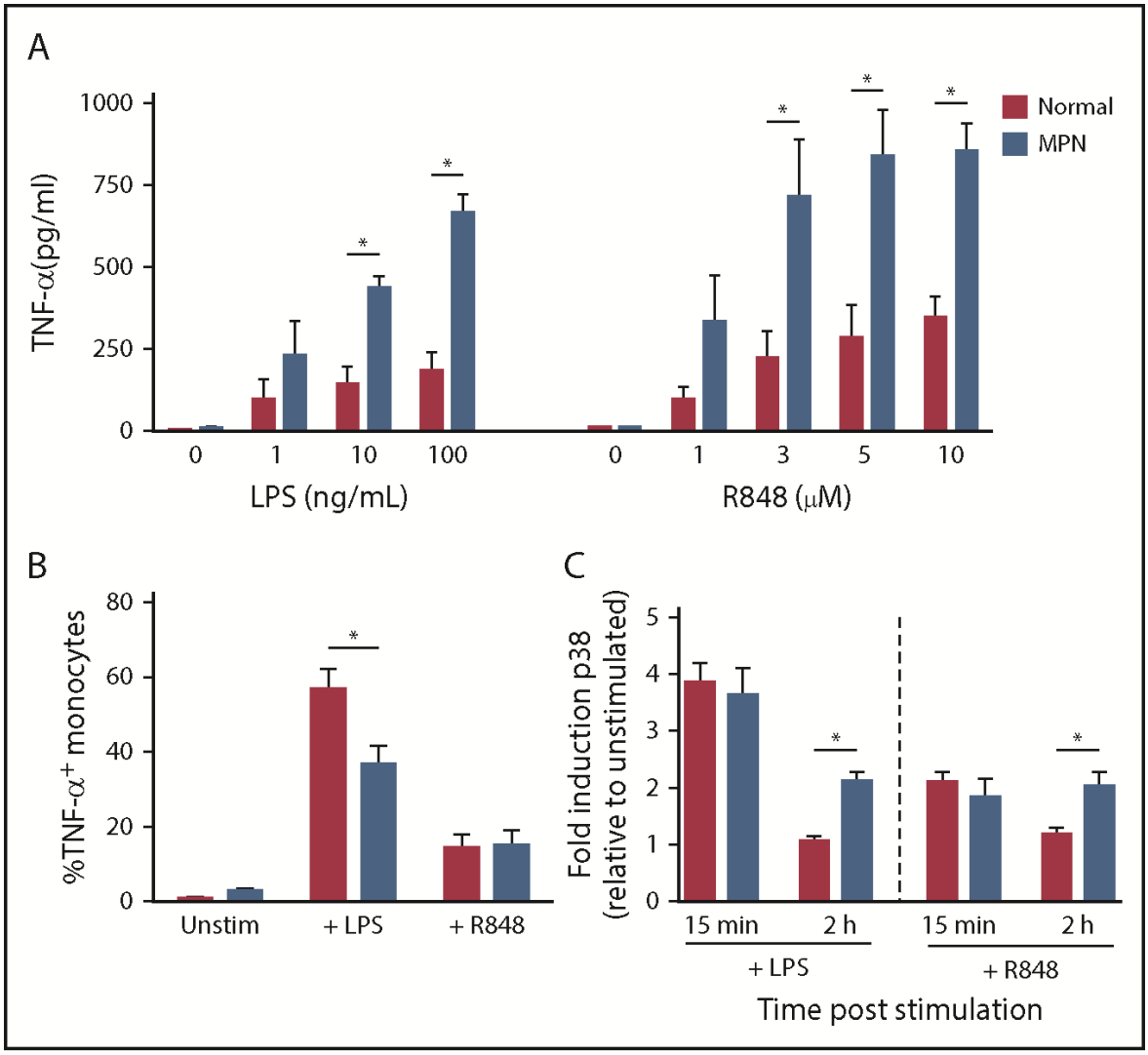


Figure 2.1 Increased TNF-α production by MPN monocytes after stimulation with TLR agonists. (A) MPN (n = 2 PV, 2 ET, and 1 MF) and normal (n = 5) monocytes were stimulated with TLR agonists LPS or R848 at the concentrations shown. After 24 hours of culture, supernatant was harvested and TNF-α was measured by ELISA. (B) MPN (n = 8 PV, 3 ET, and 2MF) and normal (n = 8) monocytes were stimulated with 10 ng/mL LPS or 5 μM R848 and incubated with brefeldin A for 4 hours to prevent protein export. Intracellular staining for TNF-α was performed, and cells were analyzed by flow cytometry. (C) MPN (n = 2 PV and 5 ET) and normal (n = 5) monocytes were stimulated with 10 ng/mL LPS or 5 μM R848 for 15 minutes or 2 hours before fixation and permeabilization. Cells were stained for phospho-p38 and analyzed on a flow cytometer. *$P < .05$.

Persistent production of TNF-α by MPN patient monocytes following TLR ligation

We next compared the tempo of TNF-α production in MPN versus normal controls following TLR stimulation. We stimulated monocytes with LPS and quantified TNF-α in the

supernatant at 4, 9, 18, and 24 hours later. The concentration of TNF-α at 4 hours was normalized to 1 for each patient to more easily visualize changes over time. In normal controls the concentration of TNF-α was greatest at 4 hours then consistently declined over time, but in MPN patients the concentration of TNF increased over time, peaking at 18 hours post-LPS and persisted even at 24 hours post-LPS (figure 2.2A). We also performed intracellular flow cytometry at multiple time points following LPS stimulation and found that monocytes from MPN patients maintained a higher percentage of TNF-α^+ monocytes at later time points as compared to normal controls (figure 2.2B). Our observations that the exaggerated TNF-α production is seen at late but not early time points following LPS stimulation implicates a defect in the TLR signaling negative feedback loop in MPN patients.

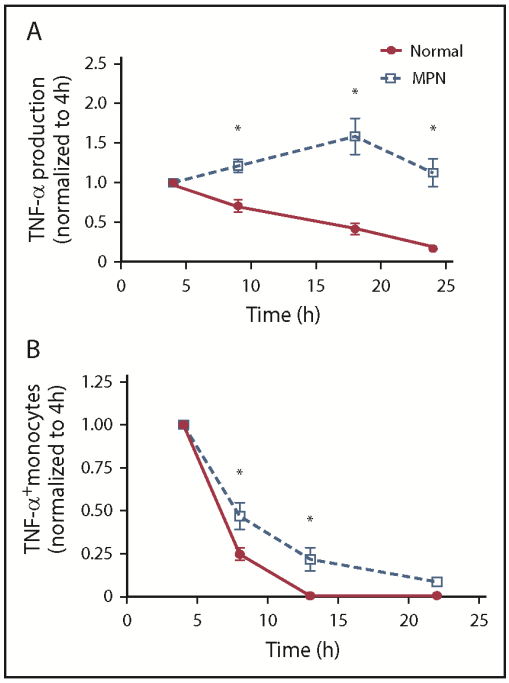


Figure 2.2 MPN monocytes persistently produce high levels of TNF-α. (A) MPN (n = 7 PV, 5 ET, and 4 MF) and normal (n = 13) monocytes were stimulated with 10 ng/mL LPS for 4, 9,18, and 24 hours before harvesting supernatant for ELISA. The amount of TNF-α produced at 4 hours was normalized to 1. (B) MPN (n = 5 PV, 4 ET, and 2 MF) and normal (n = 6) monocytes were stimulated with 10 ng/mL LPS for 4, 8, 13, and 22 hours before harvesting for flow cytometry analysis. All samples were treated with brefeldin A for 4 hours before harvesting. The percentage of monocytes expressing TNF-α at 4 hours was normalized to 1. *P< .05.

Blunted response to IL-10 by MPN monocytes is responsible for persistent TNF-α production in response to TLR ligation

IL-10 is produced in monocytes in response to LPS stimulation and acts as a negative-feedback mechanism to dampen TNF-α production [14, 17]. To test the hypothesis that MPN monocytes produce less IL-10 in response to TLR activation, we measured IL-10 production by ELISA. We found that MPN monocytes produce at least as much IL-10 as normal controls at all time points after LPS-stimulation (figure 2.3A). These data demonstrate that MPN patient monocytes produce adequate IL-10 in response to TLR stimulation and yet do not dampen TNF-α production.

We then measured the ability of recombinant human IL-10 (rhIL-10) to dampen LPS induced TNF-α production in MPN patients and normal controls. A low concentration (0.5ng/mL) of recombinant human IL-10 reduced LPS-induced TNF-α production by monocytes (figure 3B) as well as the percentage of TNF-α^+ monocytes (figure 2.3C) by greater than 50% in normal controls but only by 25% in MPN patients. However, higher concentrations of IL-10 (5, 10ng/mL) were able to reduce LPS-induced TNF-α production in normal and MPN monocytes to a similar degree. This suggests either that IL-10 produced by MPN monocytes is inherently ineffective or that IL-10R signaling in MPN patient monocytes is blunted in MPN compared to normal controls.

We measured IL-10R (CD210) cell surface expression in MPN and normal control monocytes by flow cytometry and did not find a decrease in IL-10R expression in MPN patients (figure 2.3D). To further evaluate IL-10R signaling in MPN patients, we compared phospho-STAT3 (pSTAT3) activation of MPN versus normal control monocytes in response to IL-10

stimulation (figure 2.4A). MPN monocytes did not induce pSTAT3 as robustly as normal controls in response to 10ng/mL or 50ng/mL IL-10 ($p \leq 0.01$). Because SOCS3 expression is typically upregulated in response to IL-10R activation and dampens cytokine signaling [18, 19], we also compared mRNA levels of SOCS3 in MPN versus normal control monocytes stimulated with IL-10 (figure 4B). IL-10 at high concentrations did not induce expression of the SOCS3 gene as effectively in monocytes of MPN patients as it did in normal controls. Taken together, our observations demonstrate that MPN monocytes have blunted IL-10R signaling resulting in unrestrained TLR-agonist-induced TNF-α production.

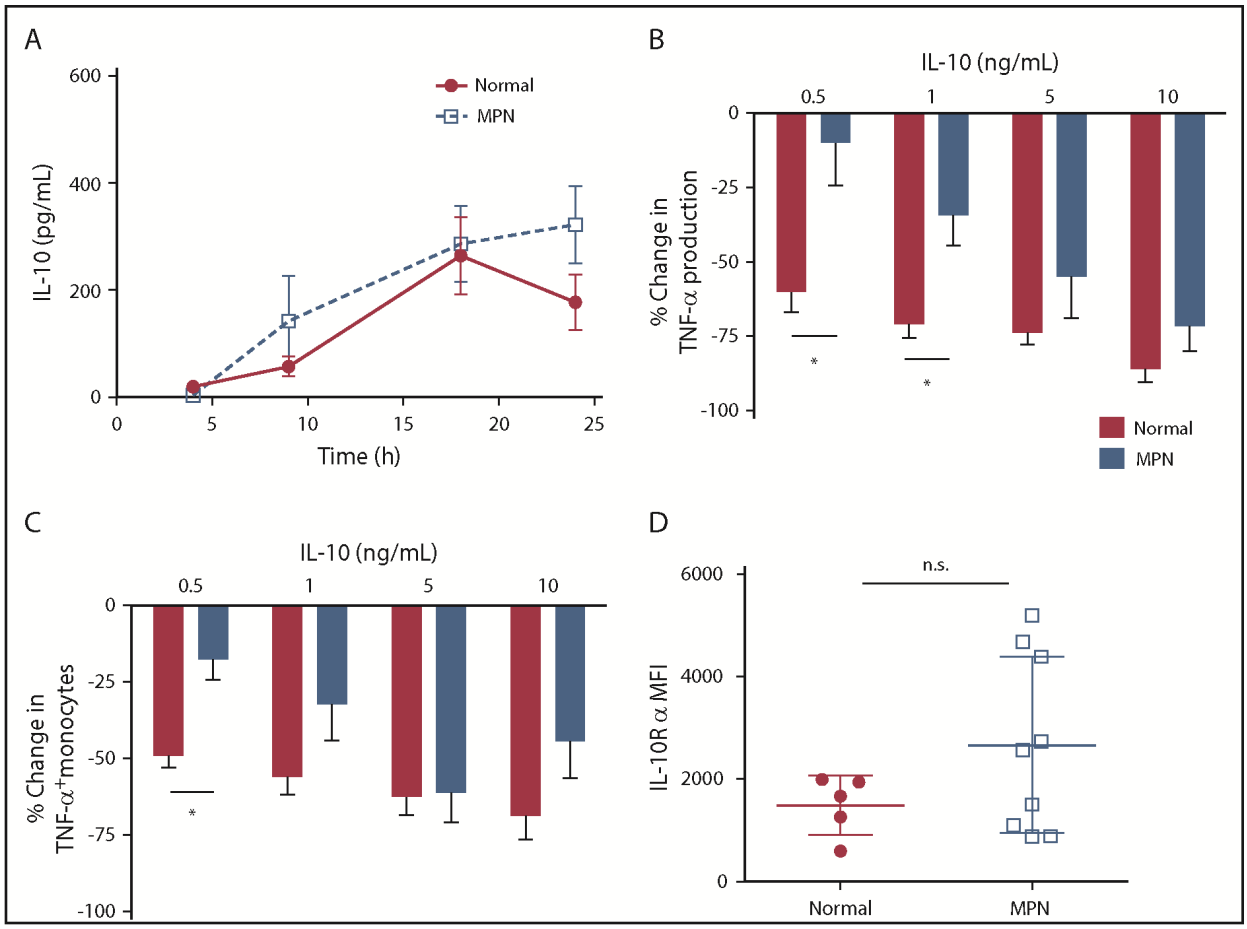


Figure 2.3. MPN monocytes produce adequate IL-10 but are less responsive to IL-10. (A) MPN (n = 4 PV, 4 ET, and 3 MF) and normal (n = 7) monocytes were stimulated with 10 ng/mL of LPS for 4, 9, 18, and 24 hours before harvesting supernatant for quantification of IL-10 via ELISA. (B) MPN (n = 2 PV and 3 ET)

and normal (n = 6) monocytes were stimulated with 10 ng/mL LPS and various concentration of IL-10 simultaneously for 4 hours between harvesting of the supernatant for ELISA. The percentage change in TNF-α is measured by the difference in TNF-α production between adding IL-10 and without IL-10. (C) MPN (n = 2 PV and 3 ET) and normal (n = 5) monocytes were stimulated with 10 ng/mL LPS and various concentrations of IL-10 for 4 hours with brefeldin A before performing intracellular staining for TNF-α. The changes in TNF-α–positive monocytes are measured by the difference in monocytes expressing TNF-α between adding IL-10 and without IL-10. (D) The mean fluorescence intensity (MFI) of IL-10 receptor α is measured by gating on MPN (n = 4 PV, 3 ET, and 2 MF) and normal (n = 5) $CD33^{high}$ $CD14^{+}$ monocytes from mononuclear cells, using flow cytometry analysis. *P < .05. ns, not significant.

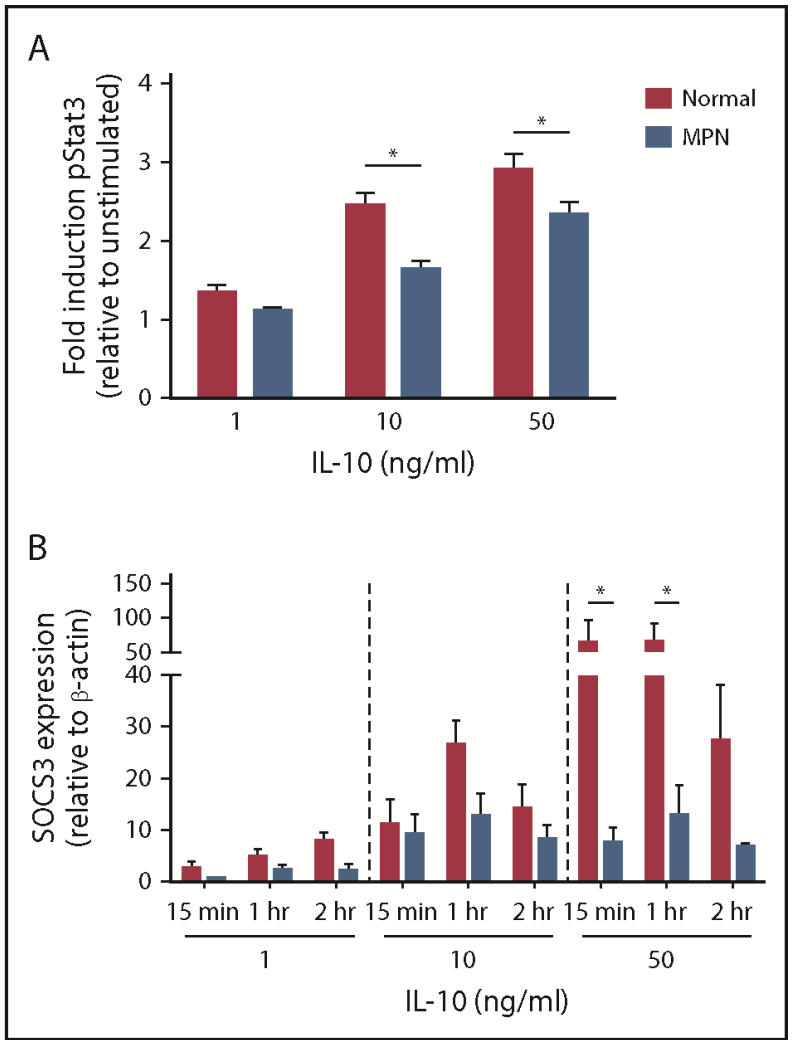


Figure 2.4. MPN monocytes have defective IL-10 signaling. (A) MPN (n = 9 PV, 6 ET, and 4 MF) and normal (n = 18) peripheral blood was stimulated for 15 minutes with IL-10 at the concentrations shown before fixation and permeabilization. $CD33^{high}$ $Cd14^{+}$monocytes were gated for pStat3 and analyzed via flow cytometry. (B) MPN (n = 2 PV and 1ET) and normal (n = 3) monocytes were stimulated with IL-10 for 15 minutes, 1 hour, or 2 hours at the concentrations shown. SOCS3 mRNA was quantified by quantitative PCR and normalized to β-actin. *P < .05.

Blockade of IL-10R signaling induces persistent TNF-α production in normal control monocytes

We inhibited IL-10R signaling in normal control monocytes using an IL-10R blocking antibody and measured the impact on LPS induced TNF-α production over time (figure 2.5A). Whereas IL-10R blockade did not have an impact on LPS induced TNF-α production at early time points (4hrs) IL-10R blockade increased LPS induced TNF-α production at later time points, confirming our expectation that blocking IL-10R in normal monocytes should induce them to produce TNF with MPN-like kinetics.

IL-10 resistance correlates with TNF-α persistence in MPN patients

We found that the degree of TNF-α persistence following LPS stimulation as well as the ability of IL-10 to dampen LPS induced TNF-α production was variable among MPN patients, with some patients being extremely abnormal and others being closer to normal. We reasoned that if TNF-α persistence is due to blunted IL-10R signaling then those patients with less of an ability to respond to IL-10 should have more extreme TNF-α persistence. For each MPN patient and normal control we calculated the TNF-α persistence score, defined as ((TNF-α at 24hrs)/(TNF-α at 4hrs)) as well as the IL-10 resistance score, defined as (TNF-α of LPS + 1ng/mL IL-10)/(TNF-α of LPS + 0ng/mL IL-10)) (figure 2.5B). We found that the TNF-α persistence score and the IL-10 resistance score had a Pearson correlation coefficient (r) of 0.72, demonstrating that the inability of IL-10 to reduce LPS induced TNF-α production correlates with an increased amount of TNF-α at 24 hours as compared to 4 hours as would be expected if IL-10 resistance is responsible for the persistent TNF-α production in MPN patients.

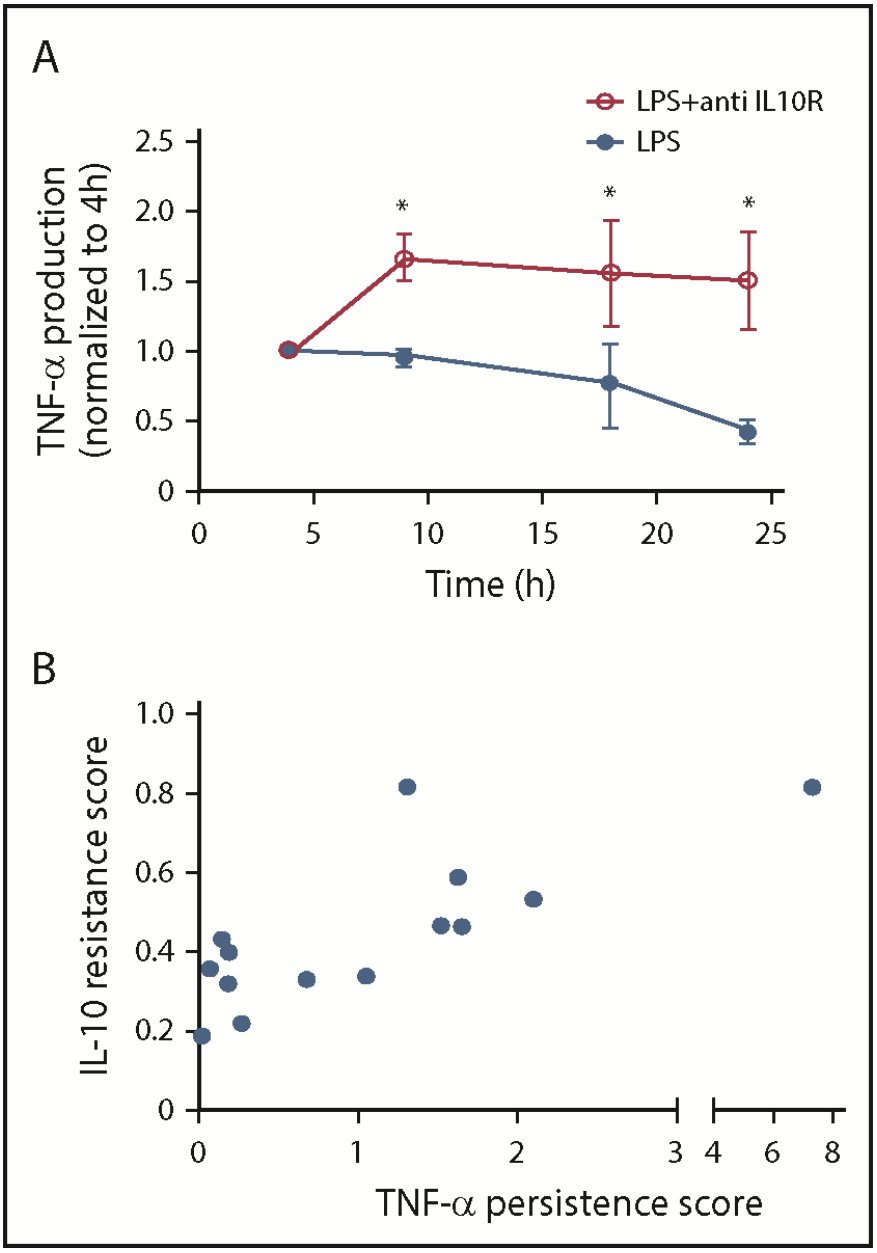


Figure 2.5. IL-10R blocking is correlated to elevated TNF-α. (A) Normal monocytes (n = 2 PV, 2 ET, and 1 MF) were stimulated with 10 ng/mL LPS and with the addition of 1 μg/mL anti-IL-10R. Supernatants were collected for TNF-α quantification by ELISA at 4, 9, 18, and 24 hours after LPS stimulation. The amount of TNF-α produced at 4 hours was normalized to 1. (B) The correlation of TNF-α persistence score in MPN monocytes (n = 14) is defined as (TNF-α at 24 hours)/(TNF-α at 4 hours), and IL-10 resistance score is defined as (TNF-α of LPS + 1 ng/mL IL-10)/(TNF-α of LPS + 0 ng/mL IL-10). Pearson r = 0.7198, R^2 = 0.5181. *P < .05.

2.4 Discussion

A chronic inflammatory state is a well-recognized feature of MPN, derangement of inflammatory cytokines drives many of the debilitating symptoms associated with the disease [4] and correlates with inferior prognosis [20]. Inflammation likely plays an active role in MPN disease progression, giving the mutant cells a selective advantage over their wild-type counterparts [8]. We find that MPN patients produce TNF-α for a prolonged period following LPS stimulation. IL-10R signaling which normally serves to dampen TNF-α production is blunted in MPN patients (figure 7) and this could explain the prolonged TNF-α production we observe in MPN patients. Interestingly, in some instances MPN patients appear to be less responsive to inflammatory stimuli initially (for example MPN have a lower percentage of TNF+ monocytes at 4 hours in response to LPS as shown in figure 2.1B, and a generalized "sluggishness" to respond to stimuli may be a feature of MPN patients.

In addition to the chronic inflammation due to dampened IL-10R signaling, it is likely that the prolonged TLR signaling we observe in MPN monocytes extends to other immune cell populations including hematopoietic stem cells (HSC). TLR agonists such as LPS induce hematopoietic stem cell cycling [21], chronic TLR signaling causes proliferative stress which exhausts HSCs [22-24]. Although IL-10's specific role in the response of HSC to TLR ligation has not been elucidated it is conceivable that IL-10 plays a direct role in the negative regulation of TLR signaling in HSC just as it does in monocytes. Dysfunctional IL-10 signaling in monocytes leads to persistent TNF-α production following TLR ligation, and dysfunctional IL-10 signaling in HSC may lead to persistence of proliferative stress in HSC following TLR ligation resulting in accelerated aging of HSC. In support of this notion, HSC from

IL-10 knockout mice have inferior long-term reconstitution potential, and addition of IL-10 to *in vitro* cultured wild-type HSC enhances their reconstitution potential [25]. Hence, subtle dampening of IL-10R signaling could theoretically lead to accelerated HSC aging not only because it induces chronic TNF-α production but also because of direct effects on the response of HSC to TLR agonists. Further studies should examine whether dampening of IL-10R signaling negatively impacts the fitness of wild-type but not *JAK2*^{V617F} HSC, affording *JAK2*^{V617F} HSC a selective advantage in this context.

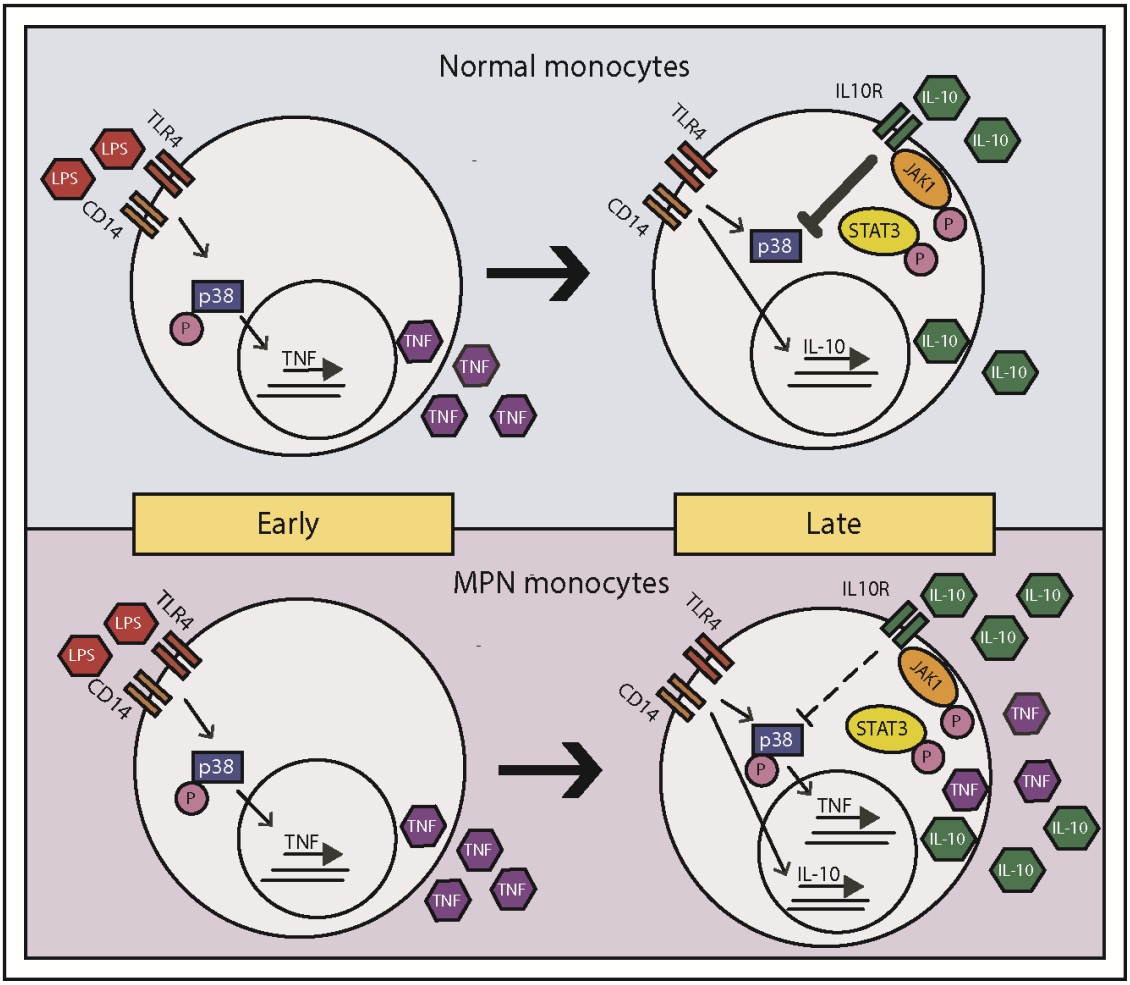
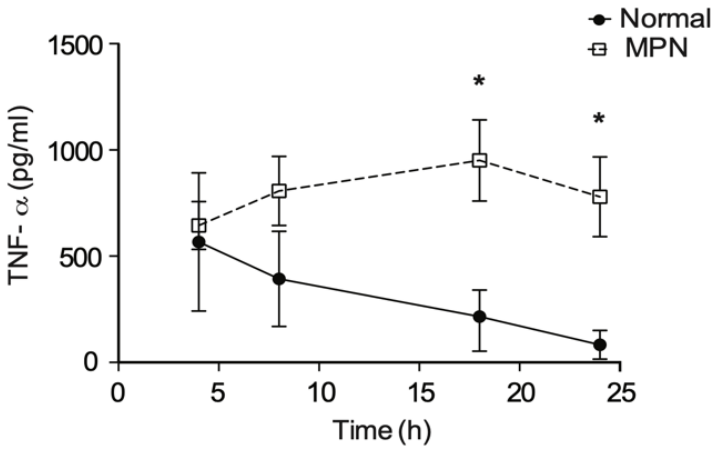
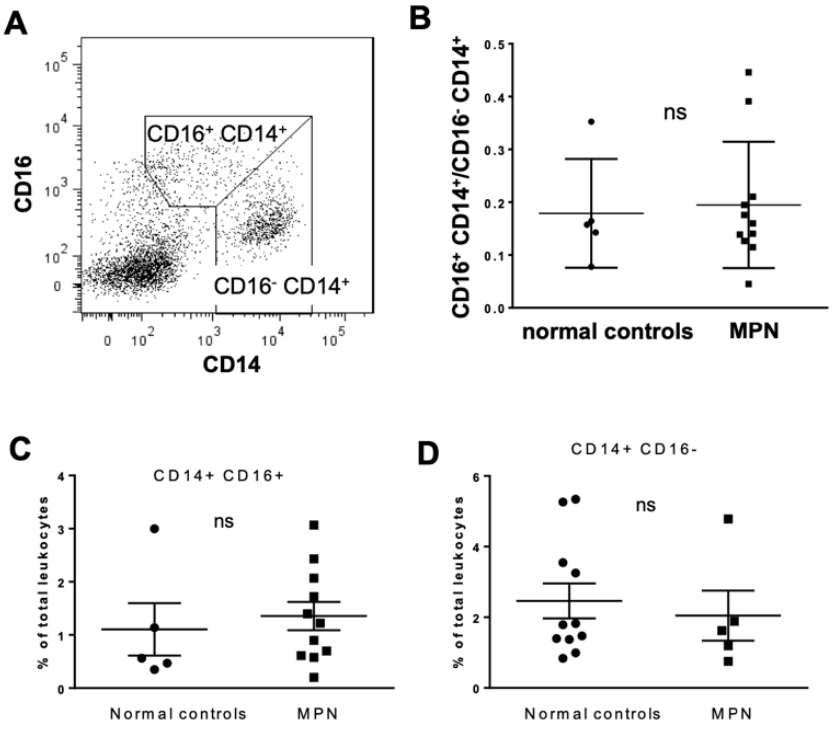


Figure 2.6 Model of LPS-induced inflammation in normal and MPN monocytes. MPN and normal monocytes produce a similar level of TNF-α in early times on LPS stimulation. Normal monocytes respond to IL-10 inhibition to abolish TNF-α production in late times, whereas MPN monocytes have a blunted response to IL-10 inhibition resulting in an overproduction of TNF-α.

2.5 Supplementary Information

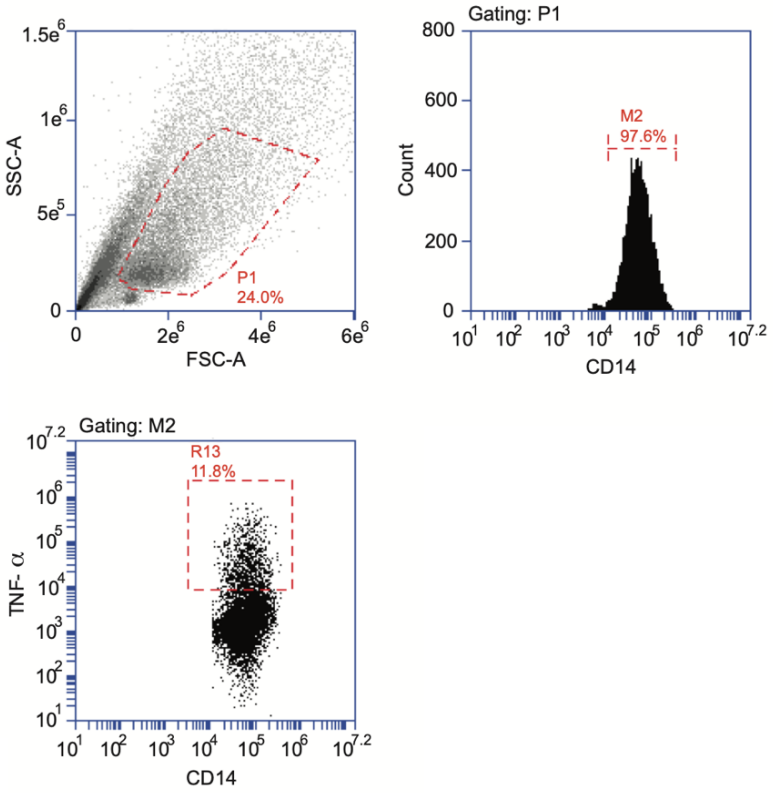


Supplemental Figure 2.1 MPN monocytes produce exaggerated TNF-α at late time point. MPN (n=16) and normal (n=13) monocytes were stimulated with 10ng/ml of LPS for 4, 9, 18, and 24h before harvesting supernatant for ELISA. Total TNF-α production is quantified.

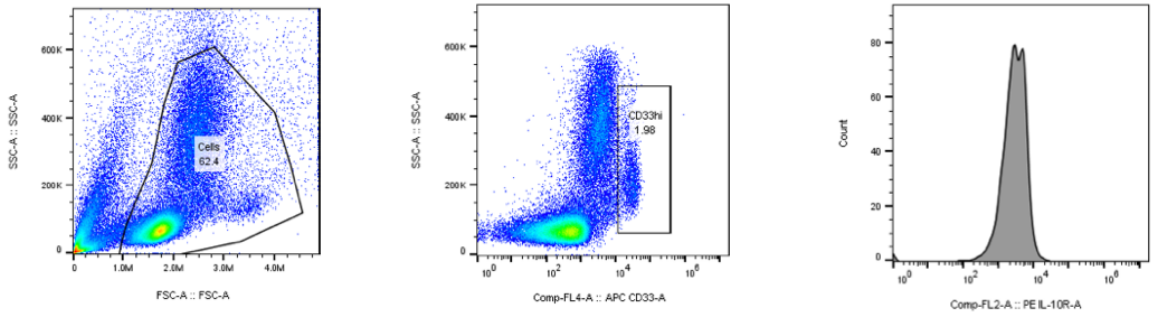


Supplemental Figure 2.2 Equivalent fraction of proinflammatory monocytes in MPN and normal controls. (A) Gating scheme for quantifying CD16+CD14+ (proinflammatory) and CD16-CD14+ monocytes. (B) Ratio of CD16+CD14+/CD16- CD14+ monocytes in MPN (n=11) as compared to normal

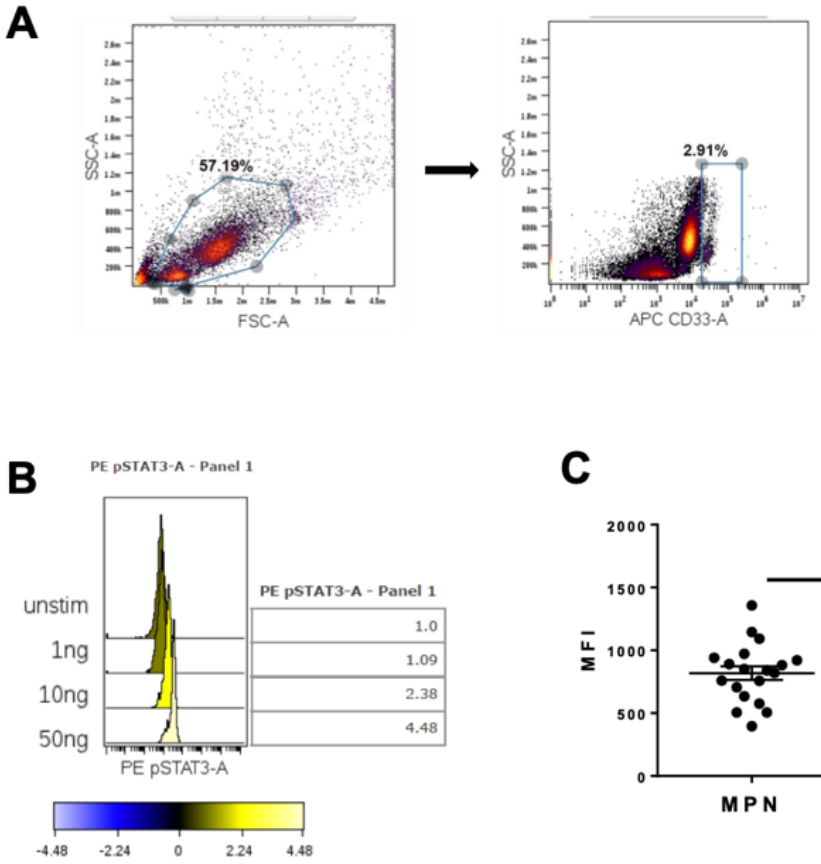
controls (n=5). (C) Percentage of CD14+CD16+ cells (of total leukocytes) is not different in MPN vs normal (D) Percentage of CD14+CD16- cells (of total leukocytes) is not different in MPN vs normal.



Supplemental Figure 2.3 Gating scheme for quantifying TNF-α+ CD14+ monocytes by flow cytometry. The P1 gate represents viable cells. The M2 gate represents CD14+ monocytes from P1, indicating that magnetic bead selected resulted in over 95% purity. The R13 gate represents the TNF-α+ population from M2, which is the percent of TNF-α+ of all CD14+ monocytes.



Supplemental Figure 2.4 Gating scheme used to measure MFI of IL-10R on monocytes. First, live cells were gated by use of FSC and SSC. Then, CD33^{hi} cells were gated, representing monocytes. Then MFI of CD33^{hi} cells was quantified.



Supplemental Figure 2.5. Analysis of pSTAT3 in normal versus MPN monocytes. (A) Gating scheme used to identify monocytes (this gating scheme was also used to identify monocytes for response to LPS and R848 phosflow). CD14 was also used as a marker, all $CD33^{hi}$ cells were in the $CD14^{+}$ gate. (B) Representative plot of data generated in Cytobank to quantify fold change pSTAT3 in response to increasing concentrations of hIL-10. C. pSTAT3 MFI of unstimulated monocytes.

Supplemental information on MPN patients used in this study

Figure 1A. TNF ELLISA at 24 hours in response to LPS and R848 (3 PV, 2 ET, 1 PMF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**splenomegaly**	**Treatment**
PV	JAK2V617F	M	70	2	Y	hydroxyurea
PV	JAK2V617F (93%)	M	68	5	Y	phlebotomy, ASA
ET	JAK2V617F	M	88	1	N	hydroxyurea
ET	JAK2V617F	M	50	<1	N	ASA
PMF	Triple negative	M	70	3	N	transfusions

Figure 1B. Intracellular TNF following LPS and R848 (8 PV, 3 ET, 2 MF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**splenomegaly**	**Treatment**
PV	JAK2V617F	F	65	20	Y	hydroxyurea
PV	JAK2V617F (40%)	M	68	4	Unknown	hydroxyurea
PV	JAK2V617F (5%)	M	50	<1	N	phlebotomy, ASA
PV	JAK2V617F (35%)	M	90	15	N	ASA
PV	JAK2V617F	M	55	<1	Unknown	ASA
PV	JAK2V617F	M	71	<1	Y	hydroxyurea
PV	JAK2V617F	M	50	2	Y	ASA, phlebotomy
PV	JAK2V617F	M	55	2	N	ASA
ET	JAK2V617F	M	55	<1	N	ASA
ET	JAK2V617F	M	68	<1	N	hydroxyurea
ET	CALR	M	45	3	N	ASA
PMF	JAK2V617F	F	72	3	Y	none
Post-PV MF	JAK2V617F	F	85	2	Y	hydroxyurea

Figure 1C. p38 phosflow (2 PV, 5 ET)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**splenomegaly**	**Treatment**
PV	JAK2V617F	F	50	3	N	ASA
PV	JAK2V617F	M	30	2	Y	ASA,
ET	JAK2V617F	F	66	1	N	Hydroxyurea, ASA
ET	JAK2V617F	M	73	10	N	hydroxyurea
ET	JAK2V617F	F	70	10	N	ASA, anagrelide
ET	CALR	F	45	4	N	ASA
ET	CALR INS	72	F	20	N	Hydroxyurea, ASA

Figure 2A. TNF ELISA timecourse (7 PV, 5 ET, 4 MF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F (14%)	F	60	7	N	Hydroxyurea
PV	JAK2V617F	F	65	10	Unknown	Hydroxyurea
PV	JAK2V617F	M	81	<1	Y	ASA
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
PV	JAK2V617F	F	49	3	N	none
PV	JAK2V617F	M	55	4	N	ASA, phlebotomy
PV	JAK2V617F (17%)	F	38	2	N	none
ET	JAK2V617F (25%)	F	83	<1	N	ASA
ET	JAK2V617F	M	58	4	Unknown	ASA
ET	JAK2V617F	F	63	10	N	PEG-IFNa, ASA,
ET	JAK2V617F	F	60	3	N	Hydroxyurea, ASA
ET	JAK2V617F	F	58	12	N	hydroxyurea
MF	JAK2V617F	F	64	3	Y	ASA
MF	CALR DEL	M	33	7	Y	None
Post-ET MF	JAK2V617F (87%)	F	68	20	Y	Chinese herbs, acupuncture
Post-ET MF	JAK2V617F	F	54	15	Y	ASA

Figure 3A. IL-10 ELISA (4 PV, 4 ET, 3 MF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since Diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	F	60	7	N	Hydroxyurea
PV	JAK2V617F	M	81	<1	Y	ASA
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
PV	JAK2V617F	M	55	4	N	ASA, phlebotomy
ET	JAK2V617F	F	60	3	N	Hydroxyurea, ASA
ET	JAK2V617F	F	83	< 1	N	ASA
ET	JAK2V617F	F	58	4	Unknown	ASA
ET	JAK2V617F	F	63	10	N	PEG-IFNa, ASA
MF	JAK2V617F	F	64	3	Y	ASA
Post-ET MF	JAK2V617F	F	54	15	Y	none
MF CALR del	CALR DEL	M	33	7	Y	none

Figure 3B. LPS + IL-10 TNF ELISA (3 PV, 3 ET)						
Type	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	F	49	3	N	none
PV	JAK2V617F (17%)	F	38	2	N	none
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
ET	JAK2V617F	F	58	4	Unknown	ASA
ET	JAK2V617F	F	58	12	N	hydroxyurea
ET	JAK2V617F (25%)	F	83	<1	N	ASA

Figure 2B. TNF intracellular staining (5 PV, 4 ET, 2 MF)						
Type	**Mutation (allele burden)**	**Sex**	**Age**	**Years Since Diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
PV	JAK2V617F	M	81	< 1	Y	ASA
PV	JAK2V617F	F	49	3	N	none
PV	JAK2V617F	M	55	4	N	ASA, phlebotomy
PV	JAK2V617F (17%)	F	38	2	N	ASA, phlebotomy
ET	JAK2V617F	F	58	4	Unknown	ASA
ET	JAK2V617F	F	60	3	N	Hydroxyurea, ASA
ET	JAK2V617F	F	63	10	N	PEG-IFNa, ASA
ET	JAK2V617F (25%)	F	83	< 1	N	ASA
MF	CALR DEL	M	33	7	Y	none
Post-ET MF	JAK2V617F	F	54	15	Y	none

Figure 3C. TNF intracellular for response to IL-10+LPS (2 PV, 3 ET)						
Type	**Mutant (allele burden**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	F	49	3	N	none
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
ET	JAK2V617F	F	58	4	Unknown	ASA
ET	JAK2V617F	F	58	12	N	hydroxyurea
ET	JAK2V617F (25%)	F	83	<1	N	ASA

Figure 3D. IL-10RA cell surface expression (4 PV, 3 ET, 2 MF)						
Type	**Mutant (allele burden**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	M	75	15	Y	hydroxyurea
PV	JAK2V617F	M	81	<1	Y	ASA
PV	JAK2V617F	M	71	4	Y	ASA
PV	JAK2V617F	M	73	20	Y	ASA, phlebotomy
ET	JAK2V617F (25%)	F	83	<1	N	ASA
ET	JAK2V617F	F	57	1	N	none
ET	CALR INS	72	F	20	N	Hydroxyurea, ASA
MF	JAK2V617F	M	65	17	Y	hydroxyurea
Post-ET MF	JAK2V617F (87%)	F	68	20	Y	Chinese herbs, acupuncture

Figure 4A. pSTAT3 phosflow (9 PV, 6 ET, 4 MF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	M	80	10	N	hydroxyurea, ASA
PV	JAK2V617F	M	81	<1	Y	ASA
PV	JAK2V617F	F	53	5	N	hydroxyurea, ASA
PV	JAK2V617F	M	55	4	N	ASA+ phlebotomy
PV	JAK2V617F	M	72	12	Y	ASA, phlebotomy
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
PV	JAK2V617F	F	49	3	N	none
PV	JAK2V617F	M	84	10	N	ASA
PV	JAK2V617F	M	66	8	Y	hydroxyurea
ET	JAK2V617F	F	63	10	N	PEG-IFNa, ASA
ET	JAK2V617F	F	22	<1	N	none
ET	JAK2V617F	F	58	4	Unknown	ASA
ET	JAK2V617F (25%)	F	83	<1	N	ASA
ET	CALR DEL	M	57	1	N	hydroxyurea
ET	CALR INS	F	72	13	N	PEG-IFNa
MF	MPL	F	79	2	N	none
PMF	JAK2V617F	M	72	5	Unknown	none
PMF	CALR DEL	M	33	7	Y	none
Post-ET MF	JAK2V617F (87%)	F	68	20	Y	Chinese herbs, acupuncture

Figure 4B. SOCS3 qPCR (2 PV, 1 ET)						
Disease	**Mutation (allele buden)**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F	M	81	<1	Y	ASA
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
ET	CALR INS	72	F	20	N	Hydroxyurea, ASA

2.6 References

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Chapter 3

Persistent TNF-α production is Independent of the Somatic Driver Mutation JAK2^{V617F}

3.1 Introduction

Chronic inflammation is a key feature of Myeloproliferative Neoplasms (MPNs). Several studies have demonstrated that MPN patients, including polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF), have elevated level of circulating pro-inflammatory cytokines [1-3]. Many of the constitutional symptoms in MPNs are also associated with deranged inflammation [3-6]. However, the role of inflammation in MPN pathogenesis and progress has not yet been established. It remains unclear whether the aberrant inflammation is a direct causation of the malignant clone in MPN. Although *JAK2*^{V617} is the most common somatic driver mutation in MPN that results in hyperactivated JAK/STAT pathway [7, 8], it was found that both the *JAK2*^{V617} mutant cells and the nonmalignant cells in MPNs produce abnormally high level of cytokines [9]. In addition, many evidence have shown that the *JAK2*^{V617} mutation alone is insufficient in clonal expansion [10, 11], which is a requirement for developing MPNs. Therefore, it is possible that chronic inflammation precedes clonal expansion, which gives the clonal an advantage to expand in this deranged microenvironment.

We have previously demonstrated that primary monocytes from MPN patients have aberrantly prolonged production of TNF-α in response to TLR activation and that this results from blunted IL-10R signaling [12]. In this study, we aim to address whether the excessive TNF-α production is a direct cell autonomous consequence of JAK2^{V617F} or an intrinsic innate immune feature that predates the development of MPN. First, we determined whether the *JAK2*^{V617F}mutant monocytes are the only cells responsible for driving high level of TNF-α in MPN patients. We also determined whether JAK2^{V617}monocytes cells can create an environment that induces normal monocytes cells to suppress IL-10 signaling and produce excessive TNF-α^{+}.

MPNs are a group of heterogeneity disease that is driven by many genetic and environmental factors [13-16]. It is difficult to determine whether the *JAK2*^{V617} mutation alone can initiate the disrupted immune signaling pathway in MPN patients. We use a *JAK2*^{V617} knock-in murine model of MPNs to investigate whether toll-like-receptor (TLR) agonist induces elevated TNF-α is production as seen in MPN patients. To further consider the possibility that persistent TNF-α production is a predisposition that precede clonal proliferation in MPN, we tested the response to IL-10 signaling pathway in dampening TNF-α in a pair of identical twins, one is affected with MPN (*JAK2*^{V617} positive) and one is unaffected with MPN (*JAK2*^{V617} negative). Understanding whether elevated TNF-α and blunted IL-10 signaling is an intrinsic feature of MPN will allow us to determine potential predisposition that creates a selective pressure that is advantageous for *JAK2*^{V617} mutant cells to clonally expand.

3.2 Materials and Methods

CD14^{+} Monocyte Isolation

Peripheral blood mononuclear cells (MNCs) were isolated by density gradient using Ficoll-paque PLUS (GE Healthcare) and red blood cells were lysed by ammonium chloride potassium (ACK) lysing buffer. Monocytes were selected using human CD14 MicroBeads (Miltenyi Biotec) and MACS separation column per the manufacturer's instructions. Cells were cultured in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine (PSL) and 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2 humidified incubator.

Quantitative JAK2^{V617F} allele burden

$CD14^+$ monocytes were stimulated with LPS for 4 or 10 hours and treated with BFA for the final 4 hours of stimulation. Cells were stained for TNFα as described above. TNF-α^+ and TNF-α^- populations were sorted on a FACS Aria Fusion (BD Biosciences). DNA was extracted by direct lysis in buffer (10mM Tris-HCl pH 7.6, 50mM NaCl, 6.25mM $MgCl_2$, 0.045% NP40, 0.45% Tween-20, 1mg/mL proteinase K) followed by incubation at 56°C for 1 hour and 95°C for 15 min. Allele burden was determined using the *ipsogen* JAK2 Muta*Quant* kit (Qiagen) using a LightCycler480 (Roche). Data were analyzed according to the manufacturer's instructions.

Sequencing of MPN patient and identical twin

Next-Generation Sequencing was validated and performed in the Division of Molecular Pathology, Department of Pathology & Lab Medicine, University of California, Irvine, CA. DNA was isolated from peripheral blood using a QIAamp DSP DNA Blood Mini Kit (Qiagen, GmbH). The targeted NGS libraries were prepared from 100 ng of DNA per sample, using the ArcherDX VariantPlex Myeloid (SK0123) workflow. The genes included on this panel are shown in the supplementary Table 1. The resulting libraries were sequenced on an Illumina MiSeq instrument using v2 chemistry (Illumina, San Diego, CA). Next, the FASTQ data files were analyzed on ArcherDX Suite Analysis software (v. 5.1.7) to identify SNPs, Indels, structural rearrangements, and Copy Number Variations.

Jak2^{V617F} Knock-In Bone Marrow-Derived Macrophages

The Jak2^{V617F} knock-in (KI) mouse model was a gift from Ann Mullally at Dana Farber Cancer Institute [11]. Normal C57BL/6J and Jak2^{V617F} KI mouse bone marrow cells were cultured on tissue-culture treated dishes in macrophage differentiation medium (RPMI 1640 supplemented with penicillin, streptomycin, L-glutamine, 10% FBS, and 10 ng/mL recombinant murine MCSF Peprotech) to generate bone-marrow derived macrophages (BMDMs). Cells were incubated for 6 days at 37° in 5% CO_2 in a humidified incubator then non-adherent cells were removed by repetitive washing.

Trans-well Assay

Monocytes from MPN and normal controls were isolated and paced in the bottom section of the trans-well dish that allows for transfer of soluble proteins but not cells. We stimulated the normal monocytes and MPN monocytes with LPS +/- IL-10 for 4 hours and 10 hours, and then add Brefeldin A to all well to retain cytokines being actively produced inside the cell. We performed intracellular staining and flow cytometry to quantify the percentage of TNF-α + normal monocytes that was placed in the top section of the trans-well dish. For each normal control, we will compare the percentage of TNFα$^+$ monocytes in the LPS alone condition versus the LPS + IL-10 condition, and the impact of IL-10 will be displayed as a percent reduction in TNFα$^+$ cells.

TNF-α and IL-10 ELISA

Human CD14$^+$ monocytes or murine BMDMs were stimulated with LPS (Invivogen), and recombinant human or mouse IL-10 (PeproTech) for the indicated time. For studies measuring

the impact of IL-10 or IL-10R blocking antibody on LPS induced TNF-α production, human recombinant IL-10 (Peprotech) or hIL-10R blocking antibody (BioLegend) were added to cells simultaneously with LPS. Supernatants were collected and centrifuged to remove cellular debris. Samples were flash-frozen and stored at $^{-}$80°C until quantification. TNF-α and IL-10 were measured using human or mouse Ready-SET-Go! ELISA kits (eBioscience) according to the manufacturer's protocol.

3.3 Results

Persistent TNF-α production is a feature of both wild-type and *JAK2^{V617F}* monocytes from *JAK2^{V617F}*-positive patients

To determine whether the persistent TNF-α production following TLR ligation is driven by JAK2^{V617F}, we sorted TNF-α^{+} and TNF-α^{-} CD14^{+} monocytes from *JAK2^{V617F}*-positive MPN patients at 4 and 10 hours post LPS stimulation and quantified the *JAK2^{V617F}* allele burden in each population (Table 1). We reasoned that if persistent TNF-α was driven by JAK2^{V617F} in a cell intrinsic manner then the *JAK2^{V617F}* allele burden should be higher in the sorted TNF-α^{+} population as compared to the sorted TNF-α^{-} population at 10 hours post LPS stimulation (Supplemental figure 3). We found the *JAK2^{V617F}* allele burden was similar in the TNF-α^{+} and TNF-α^{-} fractions both at 4 and 10 hours post stimulation, demonstrating that the both wild-type and *JAK2^{V617F}* mutant monocytes from MPN patients contribute to the persistent TNF-α production in MPN patients. This suggests that JAK2^{V617F} does not drive persistent TNF-α production following LPS stimulation in a cell autonomous manner. Instead, *JAK2^{V617F}* mutant cells may induce this phenotype upon neighboring cells or alternatively unrestrained TLR-

agonist-induced TNF-α production could be an intrinsic feature of MPN patients and could potentially be a predisposing factor to acquire the disease.

Patient	4 h		10 h	
	TNF-α$^-$	TNF-α$^+$	TNF-α$^-$	TNF-α$^+$
192	92.21	57.52	85.58	82.65
228	63.66	57.92	90.01	78.10
232	34.88	42.66	53.13	51.90
252	94.84	90.50	66.20	63.45
255	20.95	76.86	75.24	80.23

Table 3.1 JAK2^{V617F} allele burden in TNF-α$^+$ monocytes. MPN monocytes (n = 5) were stimulated with 10 ng/mL LPS for 4 or 10 hours in the presence of brefeldin A, and intracellularly stained for TNF-α. TNF-α$^+$ cells were sorted and JAK2^{V617F} allele burden was determined by quantitative PCR.

JAK2^{V617F} monocytes do not produce factors to alter normal monocytes to produce TNF-α

Next, we determine whether MPN monocytes creates and environment that induce normal monocytes to suppress IL-10 signaling and produce excessive TNF-α. Although we have shown that JAK2^{V617F} does not directly cause persistent TNF-α production in a cell intrinsic manner, it is possible that presence of mutant cells induces TNF-α production in bystander monocytes, potentially through secreted factors or cell to cell interaction. We use a trans-well dish to determine whether MPN monocytes secrete factors to alter IL-10R signaling in normal bystander monocytes (figure 3.1A).

If MPN monocytes secrete factors which inhibit the ability of normal monocytes to respond to IL-10, then we would expect to see less of an ability for IL-10 to reduce TNF-α production in normal when paired with an MPN sample than when paired with a normal control sample. We found that the normal bystander monocytes produce high level of TNF-α at

4 hours and is signific ally reduced by 10 hours, and this trend is the same for conditions when cultured with the same normal monocytes, different normal monocytes, and MPN monocytes (figure 3.1B). Our results demonstrated that there is no significant difference in the production from normal bystander monocytes being in culture with MPN monocytes and the same or different monocytes (figure 3.1C).

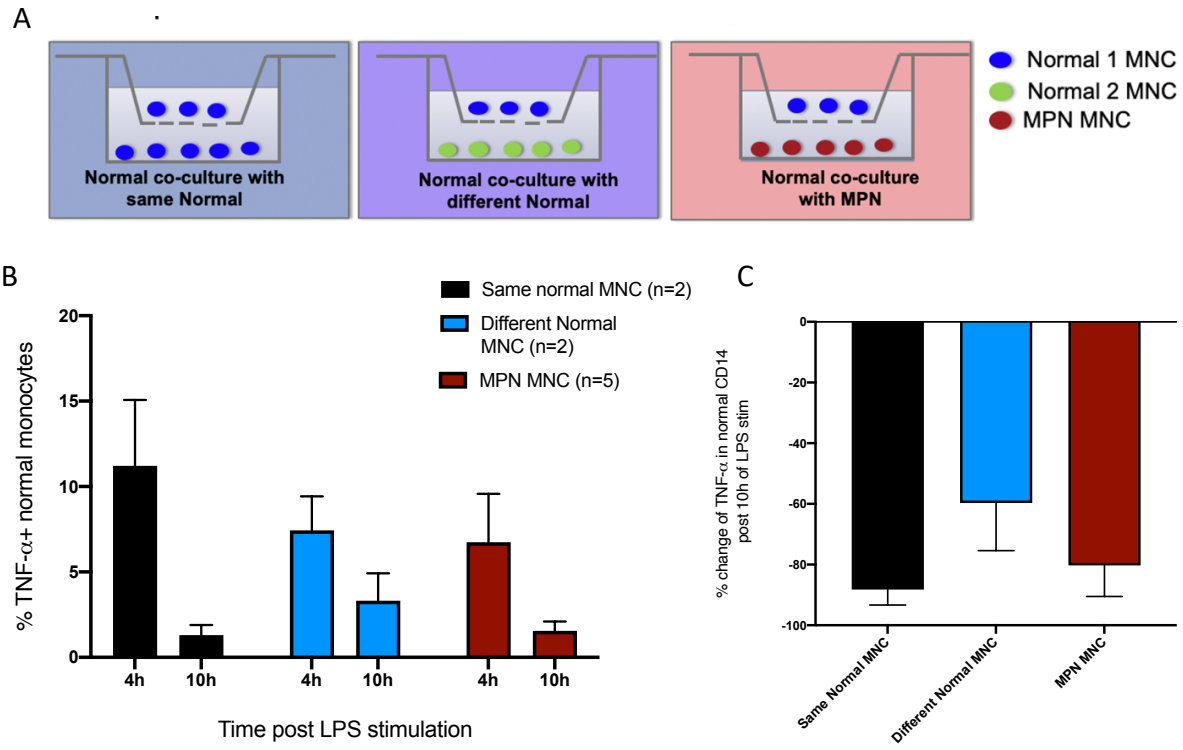


Figure 3.1 Trans-well assay used to detect whether MPN monocytes produce factor to alter normal monocytes. (A) Experimental design of the transwell assay. The normal monocytes placed on the top portion of the transwell plate is cultured with MPN and same or different normal monocytes paced on the bottom portion. Monocytes on the top portion was quantified for intracellular TNF-α. (B) The percentage of TNF-α+ normal bystander monocytes in the top section of the trans-well dish were quantified at 4 and 10 hours. (C) The percent change of TNF-α+ normal bystander monocytes from 10 hours to 4 hours were normalized.

Expression of JAK2^{V617F} does not induce persistent TNF-α production following TLR ligation

We used Jak2^{V617F} knock-in mice to more specifically test whether JAK2^{V617F} induces persistent production of TNF-α following TLR stimulation. We isolated bone marrow-derived macrophages (BMDM) from normal and Jak2^{V617F} knock-in mice and stimulated them with LPS. Intracellular cytokine staining demonstrated that Jak2^{V617F} knock-in macrophages do not produce TNF-α longer than wild-type macrophages in response to LPS stimulation (figure 3.2). Therefore, the results of our murine studies provide additional support for the notion that the TNF-α production following TLR ligation we observe in MPN patients is not directly driven by JAK2^{V617F} but instead is a unique feature of MPN patients.

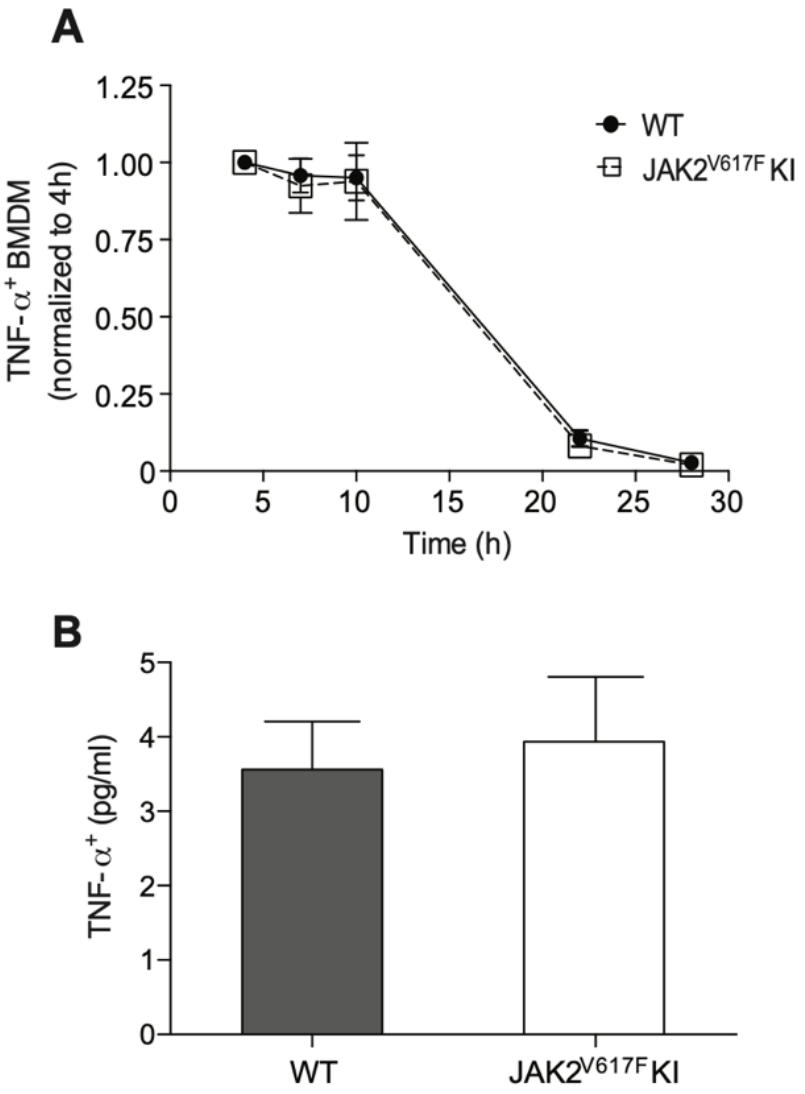


Figure 3.2 JAK2V617F knock-in mouse model does not exhibit abnormal TNF-α production. (A) Bone marrow-derived macrophages (BMDMs) from wild-type (n=6) and JAK2V617F (n=4) mice were stimulated with LPS and incubated with brefeldin A for 4, 7, 10, 22 and 28h. The percent TNF-α+ BMDMs was determined by intracellular flow cytometry. (B) TNF-α was quantified in plasma from wild-type (n=10) and JAK2V617F (n=6) mice using a high sensitivity TNF ELISA kit (Invitrogen). No significant difference was detected using unpaired t test.

Persistent TNF-α production following TLR ligation in an unaffected identical twin of MPN patient

To address whether aberrant TNF-α production is a potential genetic predisposition, we used monocytes from identical twins, discordant for MPN. One had $JAK2^{V617F}$ positive Polycythemia Vera (PV) with and the other had no evidence of an MPN (normal blood counts, no detectable somatically acquired mutations on NGS sequencing with a 75 myeloid targeted gene panel, details provided in Supplemental Table 1). We stimulated monocytes from each twin and two age and sex matched normal control with LPS and measured TNF-α and IL-10 production over time by ELISA. We found that both the PV patient and her unaffected twin had prolonged production of both TNF-α and IL-10 as compared to the normal control (figure 3.3A and figure 3.3B). We also observed that monocytes of the unaffected twin were less able to dampen LPS induced TNF-α production in response to IL-10 (figure 3.3C) than were normal monocytes. The conservation of prolonged TLR agonist-induced TNF-α production as well as blunted IL-10 response in both the PV patient and her unaffected identical twin suggests that this abnormality predates the development of MPN and is not a consequence of the $JAK2^{V617F}$ mutation. That is, the aberrant monocyte response may be an intrinsic feature of those predisposed to acquire MPN.

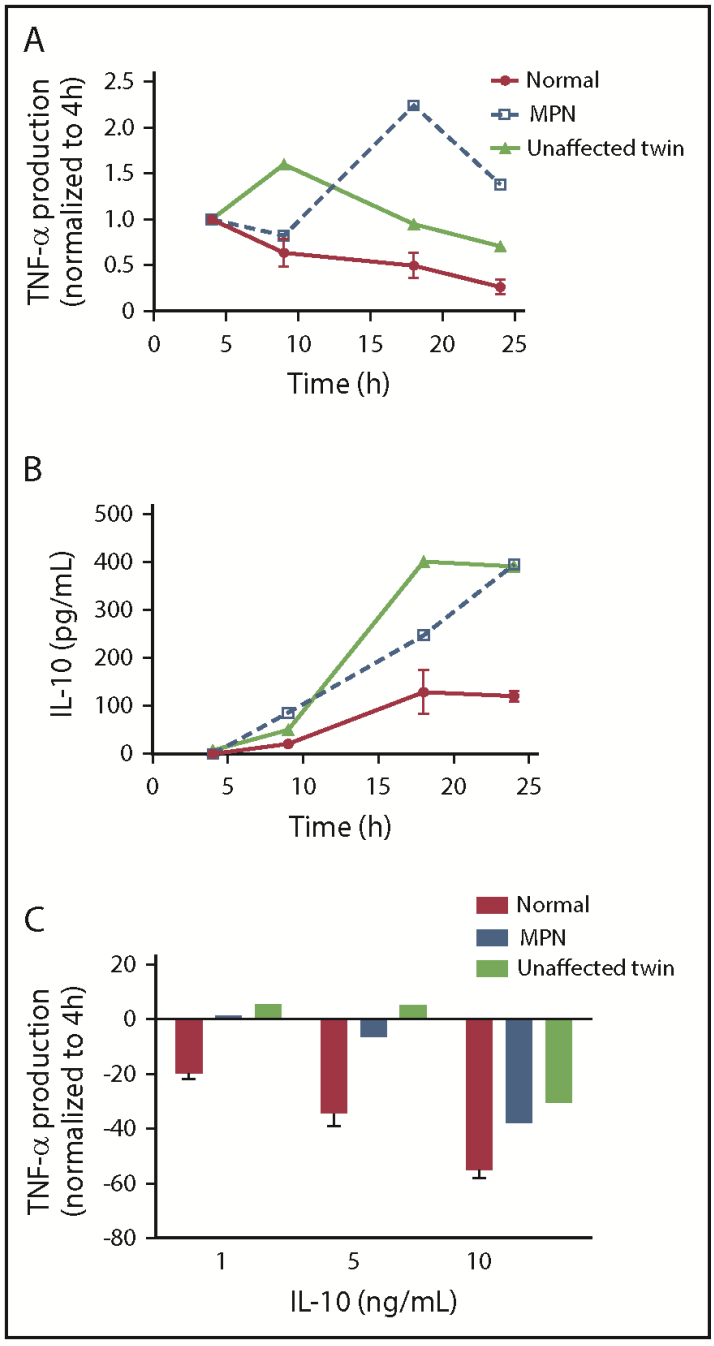


Figure 3.3 Persistent TNF-α production and IL-10R signaling defects are found in an unaffected twin of a patient with MPN. (A) The monocytes of a patient with MPN, the unaffected twin of the patient, and normal donors (n = 2) were stimulated with 10 ng/mL LPS for 4, 9, 18, and 24 hours before supernatants were harvested for ELISA. The amount of TNF-α produced at 4 hours was normalized to 1. (B) The same supernatants harvested in A were taken for quantifying IL-10. (C) The monocytes of a patient with MPN, the unaffected twin of the patient, and normal donors (n = 2) were stimulated with 10 ng/mL LPS and various concentration of IL-10 simultaneously for 4 hours between harvesting of the supernatant for TNF-α ELISA. The percentage change in TNF-α is measured by the difference in TNF-α production between adding IL-10 and without IL-10.

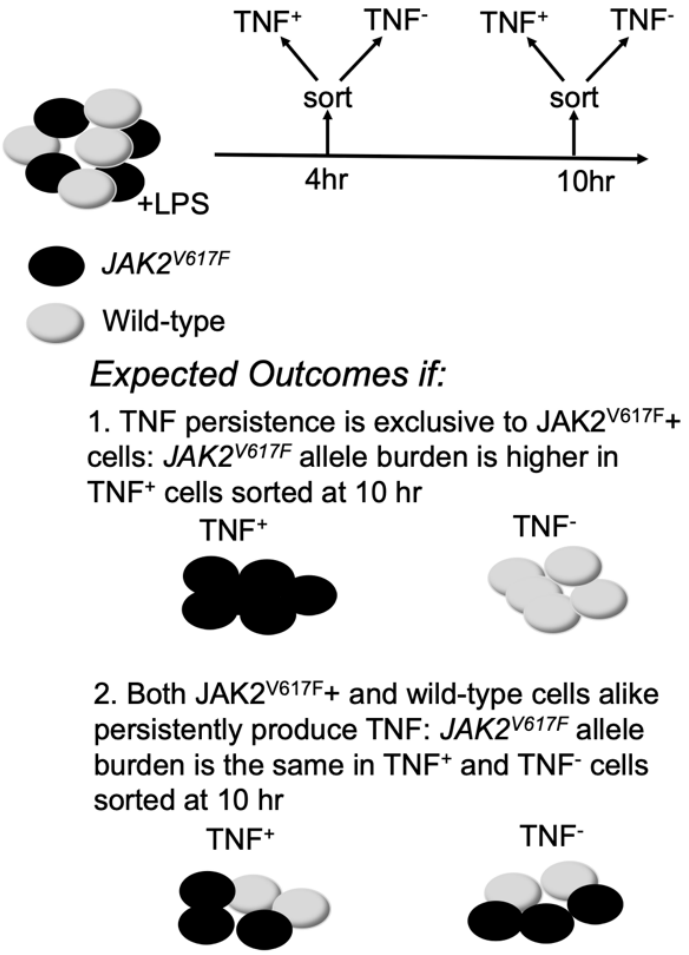
3.4 Discussion

We found that the persistent TNF-α production following LPS stimulation is not directly driven by JAK2^{V617F} in a cell intrinsic manner, as both wild-type and *JAK2^{V617F}* mutant monocytes alike from MPN patients persistently produce TNF-α after LPS stimulation (Table 1). In addition, we found that presence of an MPN clone does not induces bystander normal cells to have prolonged TNF production. Although it is clear from mouse models that the presence of *Jak2^{V617F}* cells induces inflammation [9], the specific phenotype of prolonged TNF-α production following TLR stimulation we observed in MPN patients was not recapitulated in the knock-in Jak2^{V617F} model. Furthermore, persistent TNF-α production in both a PV patient and her unaffected identical twin suggests that prolonged TLR signaling may predate the development of MPN and could possibly play a contributory role in MPN disease development.

There is a growing body of evidence linking a genetic predisposition to chronic inflammation with MPN [17] suggesting that certain types of chronic inflammation may predispose people to MPN. MPN patients and their family members have an increased incidence of autoimmune diseases [18, 19]. Genome-wide association studies (GWAS) of MPN and inflammatory diseases have identified associations with the same genes. For example, the *JAK2* single nucleotide polymorphism (SNP) rs10758669, a SNP that tags the 46/1 haplotype associated with *JAK2^{V617F}* mutated MPN [20-22], was also identified to be associated with Crohn's disease [23]. It has been proposed that the *JAK2* 46/1 haplotype results in an augmented response to cytokine stimulation leading to increased inflammation [24]. In addition, SNPs in *SH2B3* (Lnk) are associated with MPN [21] as well as multiple sclerosis [25].

We have found blunted IL-10R signaling in MPN patients as well as the unaffected twin of an MPN patient, supporting the idea that blunted IL-10R signaling may be a feature of those predisposed to acquire MPN. Genetic loss of IL-10R signaling leads to inflammatory disease in both humans and mouse models. IL-10 deficient mice develop chronic enterocolitis [26]. In humans, mutations in IL-10R cause early onset inflammatory bowel disease [27] with persistent LPS induced TNF-α production, the inability of IL-10 to reduce LPS induced TNF-α production, and failure to upregulate SOCS3. Interestingly, patients with MPN have a higher rate and absolute risk of inflammatory bowel disease [28] linking these two disease entities with potentially common predispositions. Monocytes from individuals carrying specific IL-10R variants are less sensitive to IL-10-mediated inhibition of TNF-α production, reminiscent of our results in MPN patients. It is conceivable that dampened IL-10R signaling could lead to both a predisposing factor common to both inflammatory bowel disease and MPN. Moreover, restoration of IL-10R signaling in MPN could potentially be of therapeutic benefit by normalizing excessive inflammatory cytokine production.

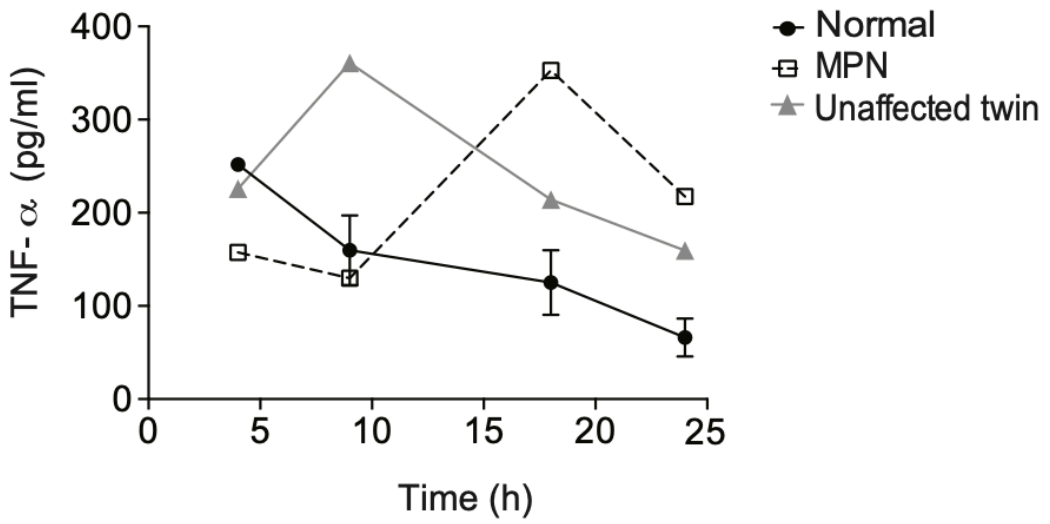
3.5 Supplementary Information



Supplemental Figure 3.1 Methods of quantifying allele prudent in TNF-α^+ and TNF-α^- fractions as shown in Figure 3.1.

Table 1. JAK2V617F allele burden (2 PV, 2 ET, 1 MF)						
Disease	**Mutation (allele burden)**	**Sex**	**Age**	**Years since diagnosis**	**Splenomegaly**	**Treatment**
PV	JAK2V617F (93%)	M	58	2	N	ASA, phlebotomy
PV	JAK2V617F (48%)	F	51	6	N	none
ET	JAK2V617F	M	80	7	N	none
ET	JAK2V617F	M	63	3	N	hydroxyurea
Post-PV MF	JAK2V617F	M	74	18	Y	hydroxyurea

Supplemental Table 3.1 Information of MPN patients used quantifying allele burden as shown in Table 3.1



Supplemental Figure 3.2 MPN patient and her unaffected identical twin produce exaggerated TNF-α at later time points as compared to normal. The monocytes of a MPN patient, her unaffected identical twin, and normal donors (n=2) were stimulated with 10ng/ml of LPS for 4, 9, 18, and 24h before supernatant were harvested for ELISA. Total TNF-α production is quantified.

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Chapter 4

Blocking IL-10R Signaling Provides a Selective Advantage for JAK2^{V617F} over Wild Type Hematopoietic Stem Cells

4.1 Introduction

Myeloproliferative Neoplasms (MPNs) is a group of blood cancer that are characterized by clonal expansion of hematopoietic stem cells (HSCs). MPNs result from the acquisition of mostly mutually exclusive somatic mutations in HSC [1], including Janus Kinase 2 (*JAK2)*, Calreticulin (*CALR*), and Myeloproliferative Leukemia Virus (*MPL*), which lead to constitutive activation of the JAK-STAT pathway and consequentially an overproduction of myeloid cells [2, 3]. The initiation of MPN pathogenesis requires clonal expansion, which is the expansion of a malignant clone that has gained a selective advantage to outcompete non-malignant cells [4-8]. Although the $JAK2^{V617F}$ mutation is the most prevalent phenotypic driver mutation in MPN, several studies have demonstrated that $JAK2^{V617F}$ HSCs do not have a selective advantage over wild-type HSCs under normal conditions [9, 10]. In addition, it was found that $JAK2^{V617F}$ impair hematopoietic stem cells function in a $JAK2^{V617F}$ knock-in murine model [11]. This evidence suggests that $JAK2^{V617F}$ alone is insufficient in driving clonal expansion, and that there must be additional factors that provide $JAK2^{V617F}$ a selective advantage.

Chronic inflammation has long known to be play a central role in the development and progression of myeloid malignancies [12-15], but how inflammation mediates cancer initiation has yet to be elucidated. The Fleischman lab has previously demonstrated that a proinflammatory cytokine Tumor Necrosis Factor alpha (TNF-α) promotes the expansion of the $JAK2^{V617F}$ malignant clone, thereby giving them a selective advantage over wild-type cells [16]. Hence, chronic Inflammation is not only responsible for many of the symptoms in MPN [17-20], but also creates an environment that favors growth of mutant over wild-type cells.

Understanding the underlying cause of clonal expansion in MPN will provide critical insights for therapeutic target for this disease.

We have previously identified a signaling defects in the IL-10 pathway that results in a persistent TNF-α production in response to Toll-Like Receptor (TLR) ligation in primary MPN patient monocytes [21]. IL-10 is potent anti-inflammatory cytokine that is dampening TLR agonist induced TNF-α production in monocytes [22-24]. This persistent TNF-α production is found in both wild-type and $JAK2^{V617F}$ monocytes from MPN patients, suggesting that $JAK2^{V617F}$ does not directly cause persistent TNF-α production in MPN patients. In addition, monocytes from a $JAK2^{V617F}$ murine model do not produce persistent TNF-α in response to TLR stimulation as we observe in human MPN patient monocytes [21]. Interestingly, we observed persistent TNF-α production and blunted IL-10 signaling in a MPN patient and the unaffected identical twin. These results suggest that impairment in IL-10 signaling may be a predisposition in MPN and play an important role in pathogenesis of this disease.

HSCs are mostly in quiescent under homeostasis to sustain their self-renewal functions. HSCs and are induced to proliferation in response to stress signal such as those from infection and inflammation, and are returned to homeostasis upon clearance [27]. Lipopolysaccharide (LPS) is a TLR4 ligand that directly induce proliferation of dormant HSCs [25]. Many studies have shown that chronic exposure to a TLR-4 ligand in a mouse model causes HSCs to continuously proliferate, resulting in a loss of HSC population [26-28]. Emerging studies have suggested that IL-10 signaling has an important role for protecting the self-renewal function of HSCs [29, 30]. We hypothesize that an impairment in the IL-10 signaling in MPN may accelerate exhaustion in wild-type HSCs and thereby, providing an opportunity for $JAK2^{V617F}$ cells to clonally expansion.

4.2 Materials and Methods

In-vivo Bromodeoxyuridne (BrdU) Incorporation Assay

C57BL/6J mice purchased from the Jackson laboratory WT and $Jak2^{V617F}$ heterozygous knock-in (KI) mice were intraperitoneally injected with LPS (Sigma-Aldrich) +/- anti IL10R antibody (BioLegend Ultra-Leaf Purified anti-mouse CD210/IL-10R antibody), isotype antibody control (BioLegend Ultra-leaf purified rat igG1), or recombinant murine IL-10 (PeproTech) for each group of day 1, day 2, and day 3 before injecting all groups with Bromodeoxyuridine (BrdU) on day 1. Bone marrow of all groups was harvested on day 0. Pacific Blue anti-mouse Lineage Cocktail, APC/Fire 760 anti-mouse CD117 (C-kit), Brilliant Violet 650 Sca-1, PE antimouse CD-150 (SLAM), and PerCP/Cyanine5.5 anti-mouse CD-48 conjugated antibody (BioLegend) were stained identify HSC population. APC BrdU Flow kit (BD Pharmingen) was used to identify $BrdU^{+}$ cells according to the manufacture's protocol. Cells were analyzed on a Novocyte Advanteon Flow Cytometer.

Competitive Transplantation Assay

C57BL/6J mice purchased from the Jackson laboratory were used as recipients for transplantation were sex match and age matched in the experiment. The $Jak2^{V617F}$ heterozygous knock-in (KI) murine model was a gift from Ann Mullally at Dana Farber Cancer Institute. Whole bone marrow of 1:1 equivalent HSC from each competing donor was quantified as indicated antibody staining scheme listed in above section and quantified in Novocyte Advanteon Flow Cytometer. Recipients were lethally irradiated using

an X-ray irradiator at 850mGy, and then rest for 16 hours before transplantation. Retroorbital transplantation was preformed using a total of 2 million whole bone marrow cells from donor competitors into irradiated recipients.

Chronic treatment of liposaccharide (LPS) and IL-10 receptor blockade

For WT:WT competitive transplantation assay, 5μg of LPS (Sigma A-Aldrich) and or 0.1mg of anti IL-10R antibody (Biolegend Ultra-Leaf Purified anti-mouse CD210/IL-10R antibody) in Phosphate-buffered saline (PBS) or PBS alone were injected interperitoneally per mouse (average 25g) weekly for 2 weeks, 1 month and 2 month (for separate experiment) before transplantation. For VF:WT competitive transplantation assay, weekly treatment of LPS and IL-10R antibody started 2 months post transplantation.

Quantification of peripheral blood cells, platelet counts, white blood cells count, histology and HSCs in the bone marrow.

Mouse anti-CD 45.1 and CD 45.2 antibody (Biolegend) were used to quantify hematopoietic cell populations. Scil Vet abc hematology analyzer was used to obtain the complete blood cell count and platelet count from mouse peripheral blood. Upon termination of the transplant, spleens and femurs of transplanted mice were collected and fixed in formalin. Hematoxylin and eosin (H&E) staining were performed for spleen and bone marrow sample. HSCs were quantified using antibody as stated in above section.

4.3 Results

IL-10R blockade prolongs the cycling of HSCs in TLR4 ligation induced proliferation

First, we examine how IL-10R blockade impact the proliferation of WT HSCs in response to LPS stimulation (figure 4.1). We hypothesized that IL-10 serves to curtail TLR signaling in HSPC just as it does in monocytes. Therefore, we assessed the tempo of HSC cycling in mice treated with LPS versus mice treated with LPS + IL-10R blocking antibody. We performed IP injection with 5μg of LPS per mouse for 3 groups (day 1, day 2 and day 3) and with LPS + 0.1mg of anti IL-10R for another 3 groups for same day interval. We injected BrdU to all groups at day 1 to label actively dividing cell and harvested the bone marrow at day 0 to measure BrdU positive HSPC marked by $Lin^{-}C\text{-}kit^{+}Sca\text{-}1^{+}$ (LKS) and HSC marked by $Lin^{-}C\text{-}kit^{+}Sca\text{-}1^{+}CD150^{+}CD48^{-}$ (LKS SLAM) surface staining (Supplemental figure 4.1).

We found that HSCs proliferate at average 10% at baseline and reached peak proliferation of average 30% at 1 day post LPS stimulation (figure 4.1B). WT HSCs has reduced back to 10% of proliferation similar to the baseline by day 3 post LPS stimulation, and this trend is the same for LPS with the addition of isotype antibody control (figure 4.1B). Next, we assessed the time course of HSC cycling in mice treated with LPS versus mice treated with LPS + IL-10R blocking antibody. LPS treatment with IL-10R blockade induce HSCs to continuously cycle at day 3 to the same extent as at day 1 (figure 4.1B). This suggests that dampening of IL-10R may accelerate inflammation induced exhaustion of HSC by increasing the time of cycling. Lastly, the addition of recombinant IL-10 cytokine and LPS treatment reduce HSCs cycling at day 1 compared to LPS alone (figure 4.1B). We found the same trend for HSPCs as we observed in

HSCs (Supplemental figure 4.2). Consistently, the percentage of hematopoietic stem and progenitor cells (HSPCs) in bone marrow post 3 days of treatment of LPS and IL-10R blockade is significantly elevated compared to LPS treatment alone (figure 4.1C). Together these results demonstrate that the addition of IL-10 blockade to LPS induce stress cause HSCs to persistently cycle and unable to return to homeostasis.

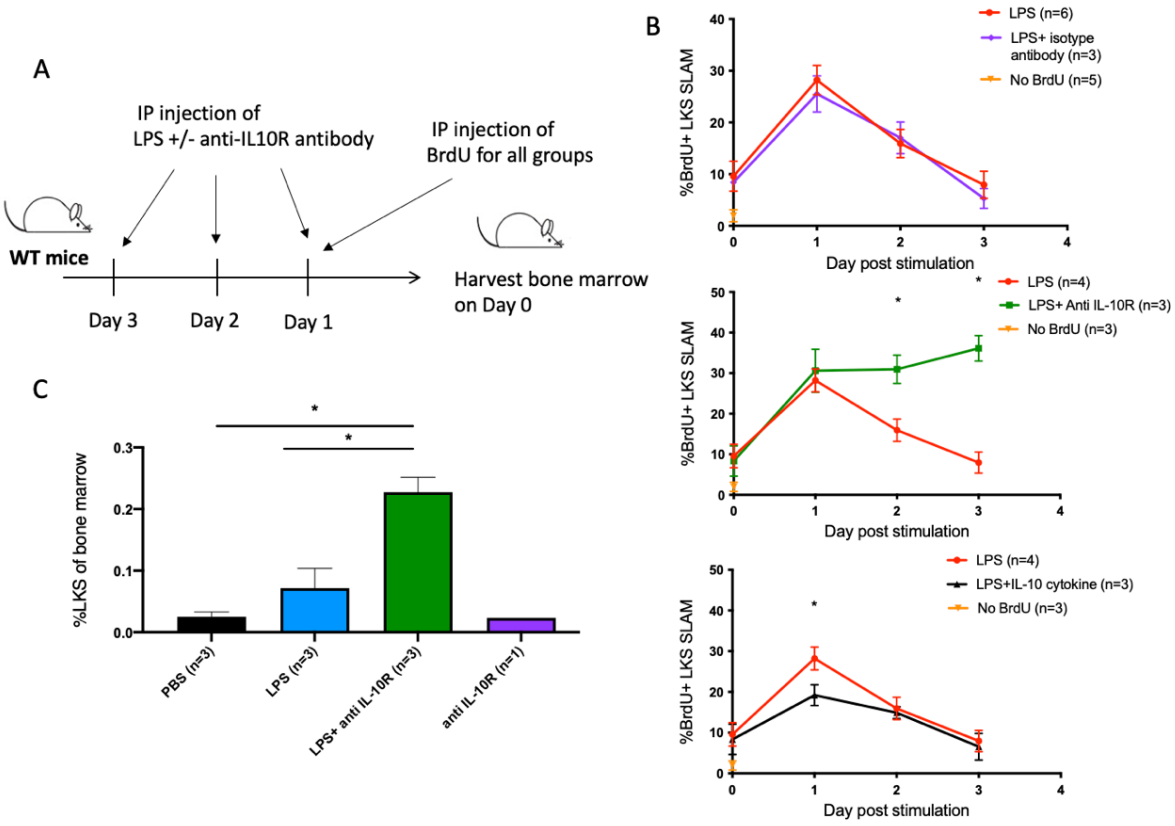


Fig 4.1 Blockade of IL-10R extends cycling of HSC following LPS stimulation. (A) Experimental design for *in-vivo* BrdU incorporation assay. (B) Percent of BrdU^{+} HSCs were quantified by LKS SLAM markers for the group of LPS only, LPS+ isotype antibody, LPS+ anti IL-10R, and LPS + IL-10 cytokine. No BrdU condition is the untreated group. (C) Percent of HSPCs of the bone marrow were quantified by LKS markers for day 3 post injection groups.

Chronic blockade of IL-10R leads to reduced fitness in competitive transplant experiments.

To determine how chronic blockade of IL-10R signaling impair HSC fitness, we performed a competitive transplantation assay to compare chronic treatment with LPS, IL-10R blockade, LPS, LPS+IL-10R with PBS as control group (figure 4.2 A). We first treated CD45.1 mice weekly with IL-10R blocking antibody (0.1mg IP) or PBS for 2 months. The bone marrow from CD45.1 treat mice was transplanted at a 1:1 ratio with age/sex matched bone marrow from unmanipulated CD45.1/2 mice and transplanted into CD45.2 lethally irradiated hosts. The repopulation of IL-10R exposed and unexposed cells were quantified overtime in peripheral blood. We found that the IL-10R blockade group has significantly reduced repopulation blood cells compared to the control group (figure 4.2B). In a separate experiment, we treated CD45.1 mice weekly with LPS (5μg IP) or PBS for 1 month before bone marrow transplantation. We found that the LPS treated group has significantly reduced repopulation of blood cells by 2-fold compared to the control group (figure 4.2C).

Next, we investigate the impact of chronic treatment of LPS alone, IL-10R blockade alone, and LPS +IL-10R blockade for a shorter time course of 2 weeks in a completive transplantation assay as described above. We found that there is no significant difference between the LPS treatment group and the PBS control group (figure 4.2D). IL-10R blockade group has a trend of reduce repopulation, but it is not significantly different from the PBS control group (figure 4.2D). Strikingly, we found that the combination of LPS and IL-10R blockade results in a drastically reduced repopulation of blood cells compared to the PBS control group (figure 4.2D). Consistently, we found that the LPS + IL10R blockade treatment significantly reduced HSCs in the bone marrow compared to the unmanipulated control but not

in other groups (figure 4.2E). These results suggest that chronic LPS treatment with IL-10 blockade negatively impact HSCs function, resulting in significantly reduced repopulate of blood cells in a relatively short time period.

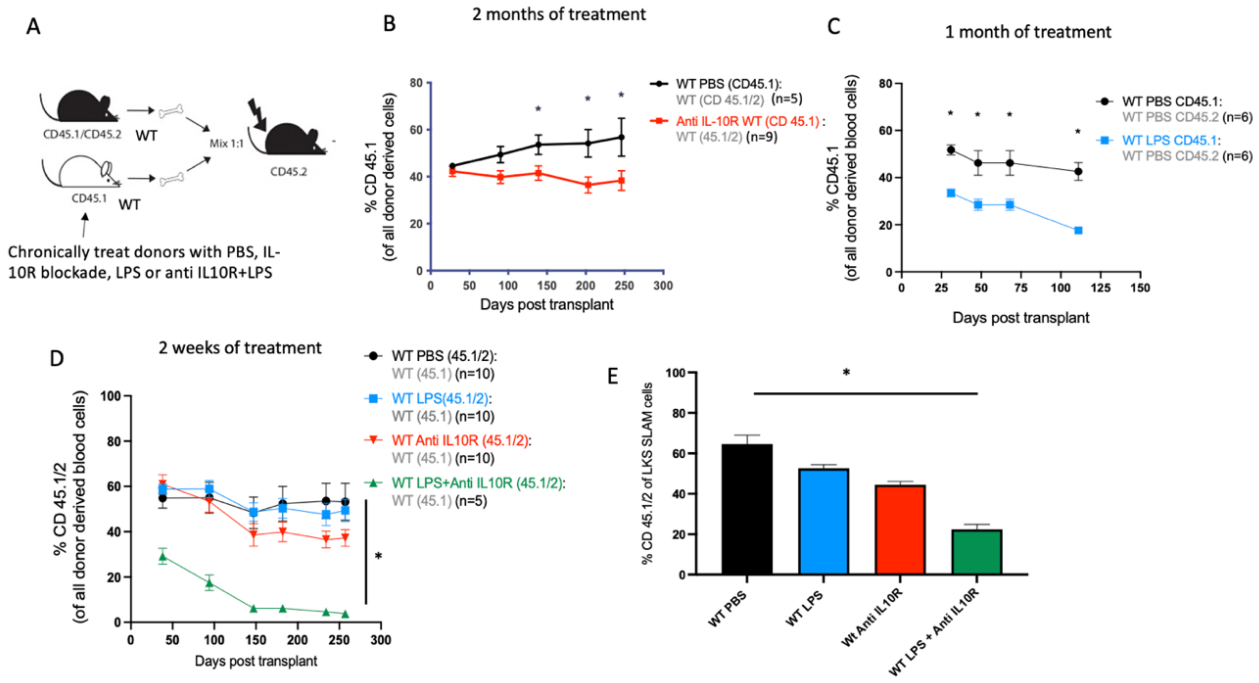


Fig 4.2 Blockade of IL-10R hastens inflammation induced exhaustion of HSCs. (A) Experimental design of competitive transplantation assay of 1:1 WT treatment group (PBS control, anti- IL-10R antibody, LPS, and LPS+ IL-10R antibody) and unmodified WT whole bone marrow cells in irradiated recipients. (B) Quantification of repopulated donor blood cells from 2 months of treatment in PBS control and IL-10R blockade group over time post transplantation. (C) Quantification of repopulated donor blood cells from 1 months of treatment in PBS control and LPS group over time post transplantation. (D) Quantification of repopulated donor blood cells from 2 weeks of treatment in PBS control, IL-10 blockade only, LPS only, and LPS+ IL-10 blockade group over time post transplantation. (E) Quantification of HSCs in the bone marrow from treatment group accordingly.

IL-10R blockade impairs wild-type HSCs more than JAK2^{V617F} HSCs

Next, we compare the proliferation of JAK2^{V617F} and WT HSCs and HSPCs using BrdU incorporation assay. As expected, LPS treatment induced cycling of HSCs at day 1 and cycling returned to that of unstimulated by day 3, while the addition of IL-10R blockade to LPS

treatment cause HSCs to continue to proliferate day 3 (figure 4.3 A). Interestingly, JAK2^{V617F} HSCs is proliferating more than WT HSCs at baseline without LPS treatment, and that the addition of IL-10 blockade and LPS did not have significant impact on their proliferation (figure 4.3 B). We observed the same trend in WT and JAK2^{V617F} HSPCs marked by LKS cells (Supplemental figure 4.3). This result suggests that JAK2^{V617F} HSCs normally proliferate more than WT HSCs, and thereby JAK2^{V617F} HSCs is more prone to HSCs exhaustion and that may lead to have less selective advantage compared to WT HSCs. In the presence of stress induce inflammation such as LPS stimulation, both JAK2^{V617F} and WT HSCs are proliferating, and thus JAK2^{V617F} has no significant selective advantage. However, in the presence of LPS and IL-10 blockade, WT HSCs are induced to proliferation at a significantly higher level than WT HSCs, which cause WT HSCs to reduce in fitness compared to JAK2^{V617F}. We tested this hypothesis in a competitive transplantation assay.

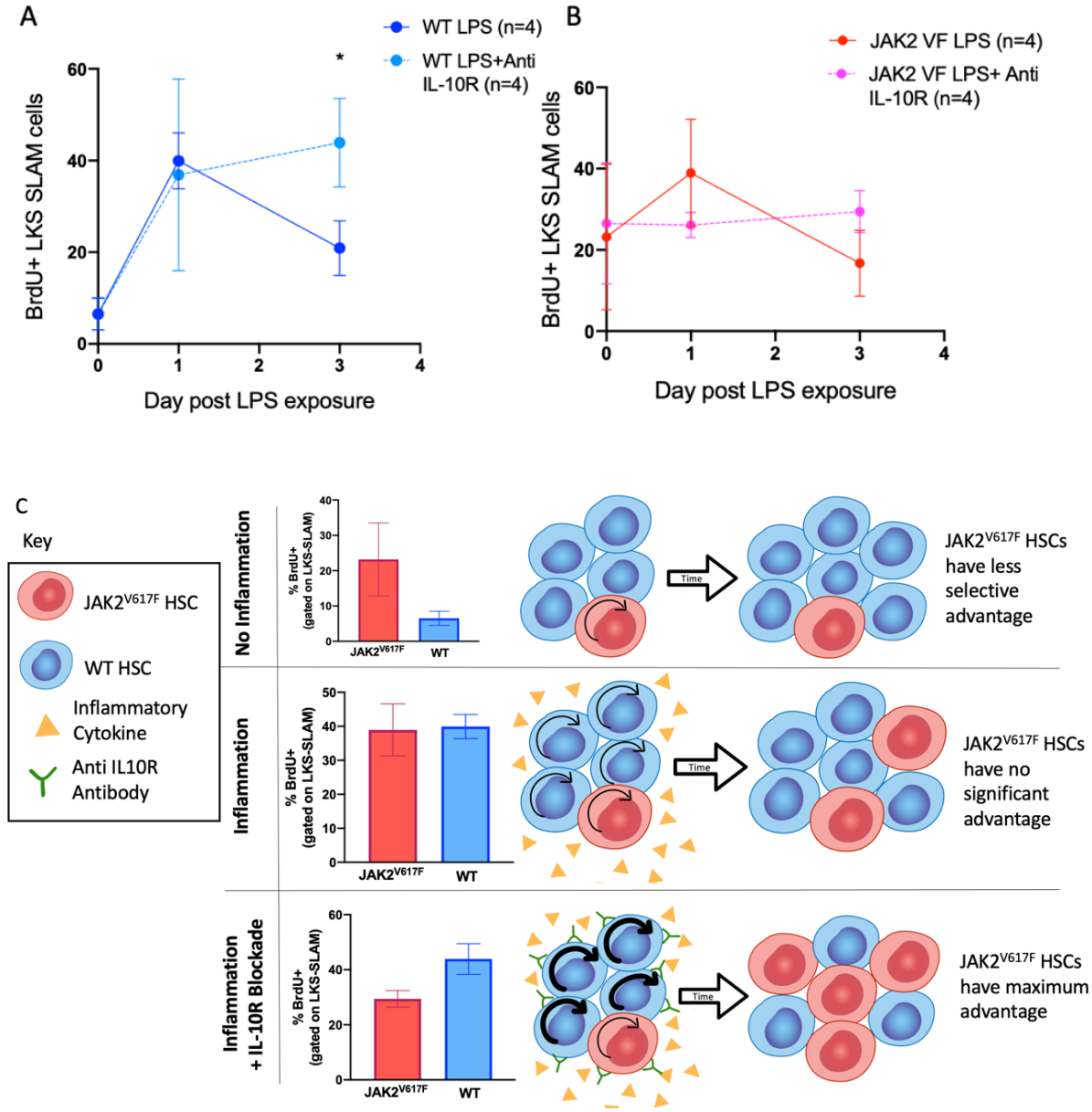


Fig 4.3 JAK2^{V617F} HSC are less impacted by IL-10R. (A) Percent of BrdU^{+} WT HSCs were quantified by LKS SLAM markers for the group of LPS only and LPS+ anti IL-10R. (B) Percent of BrdU^{+} *Jak2*^{V617F} HSCs were quantified by LKS SLAM markers for the group of LPS only and LPS+ anti IL-10R. (C) Data summary and hypothesis model of WT and *Jak2*^{V617F} HSCs cell cycling in the condition of no inflammation, LPS induced inflammation, and LPS+ IL-10R blockade.

IL-10R blockade enhances the competitive ability of Jak2^{V617F} mutant cells in transplantation assays

To evaluate the functional impact of IL-10 blockade for JAK2 ^{V617F} and WT HSCs, we performed the competitive transplantation assay using 1:1 HSC equivalent whole marrow cells of WT and JAK2 ^{V617F} donor competitors. We treated both group with anti IL-10R and control 60 days post transplantation. As expected, the untreated group had a steady decrease of JAK2 ^{V617F} peripheral blood cells over time (figure 4.4 A). In contrast, the treatment group has significantly increased JAK2^{V617F} peripheral blood cells after 120 days of treatment (figure 4.4 A). As a control, we also had a competitive transplant with an equal amount of ratio of WT (CD 45.1) and WT (CD 45.2) whole bone marrow and found that the IL-10R blocking antibody had no effect on the ability of WT cells to compete with WT cells (figure 4.4 A). After 186 days, we sacrificed both group of mice and quantified HSCs found a significant increase of HSC and HSPC in the anti IL-10R treatment group compared to the control group (figure 4.4 C and figure 4.4 D). We also found that blocking IL-10 signaling resulted in an increase of JAK2^{V617F} of donor derived HSPCs (figure4.4 E). The anti IL-10R treatment group had larger spleens compared to the untreated group (figure 4.4 B). Collectively, these results suggest that blocking IL-10 signaling results in an expansion of JAK2^{V617F} mature blood cells as well as JAK2^{V617F} HSCs and HSPCs at the expand of WT cells.

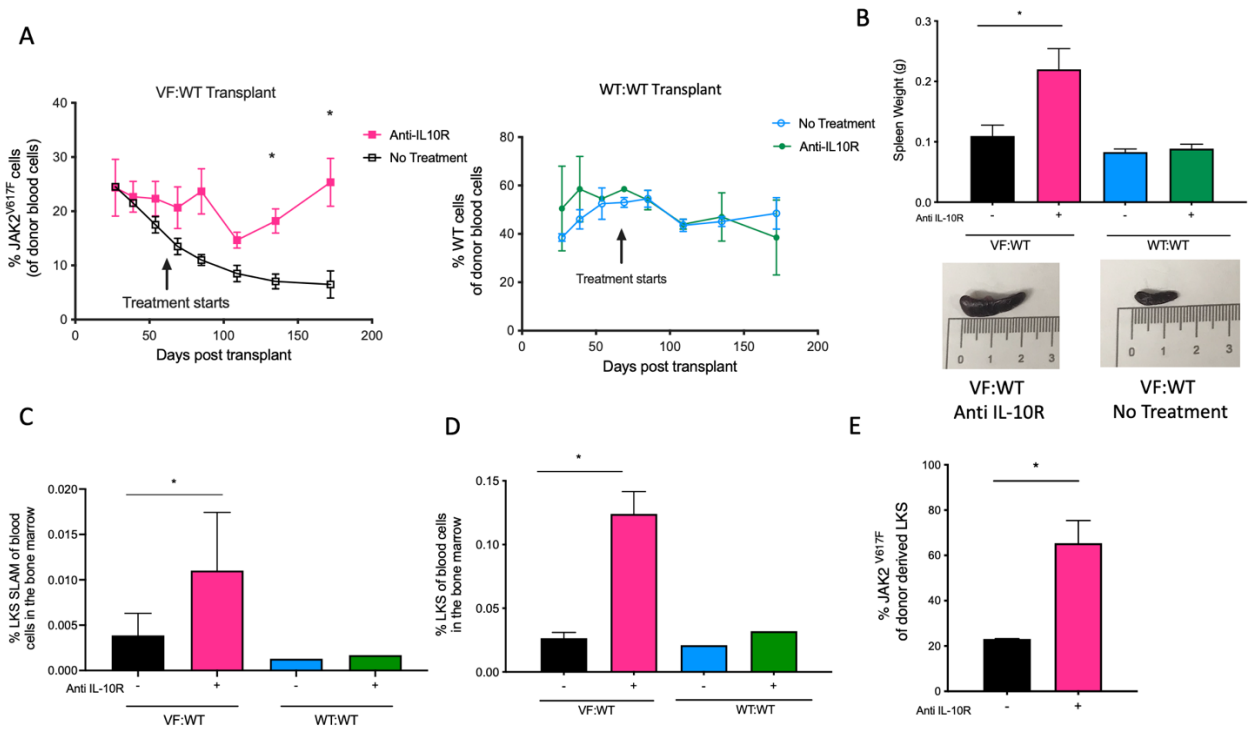


Fig 4.4 IL-10R blockade selects for JAK2^{V617F} cells. (A) Exposure to anti IL-10R started after 60 days post competitive VF:WT transplantation, and the percent of CD45.2^{+} (JAK2^{V617F}) was quantified from peripheral blood. WT:WT transplantation as control on the left (B) Spleen weight and representative images of spleen harvested after competitive transplantation of JAK2^{V617F} and WT with and without anti IL-10R. (C)Transplant was terminated on day 186 and each group were quantified for %HSCs in the bone marrow. (D) % HSPCs in the bone marrow. (E) % JAK2^{V617F} leukocytes of donor derived HSCs.

IL-10 Blockade exacerbates MPN disease pathology and progression

We have previously determined that IL-10 blockade results in higher JAK2^{V617F} repopulation in a transplantation assay. To follow up with how IL-10 blockade affects MPN disease phenotype, we quantified platelets and whole blood cell count from another transplantation assay of 1:1 ratio of JAK2^{V617F} and WT whole bone marrow (figure 4.5A). We started the anti-IL-10R antibody treatment 49 days post transplantation (0.1mg and 0.3mg IP) and ended at 291 days post transplantation. We found that both the platelets and white blood cells were significantly higher in both the low and high dose of IL-10 blockade, which

indicate a more severe MPN phenotype (figure 4.5 B and figure 4.5C). We did not observe any difference in platelets and white blood cell count before the treatment at day 36 and during recovery period after treatment ended on day 340 (supplemental figure 4.3 A-C). This indicates that active IL-10 blockade is required to augment the MPN phenotype. We also found that there was increased infiltration of megakaryocytes in both bone marrow and spleen of the IL-10 blockade group (figure 4.5D). These results show that blocking IL-10 not only results in an expansion of $JAK2^{V617F}$ cells at the expands of WT cells, but also cause an exacerbated MPN disease phenotype characterized by the presence of increased platelets, white blood cells, and megakaryocytes.

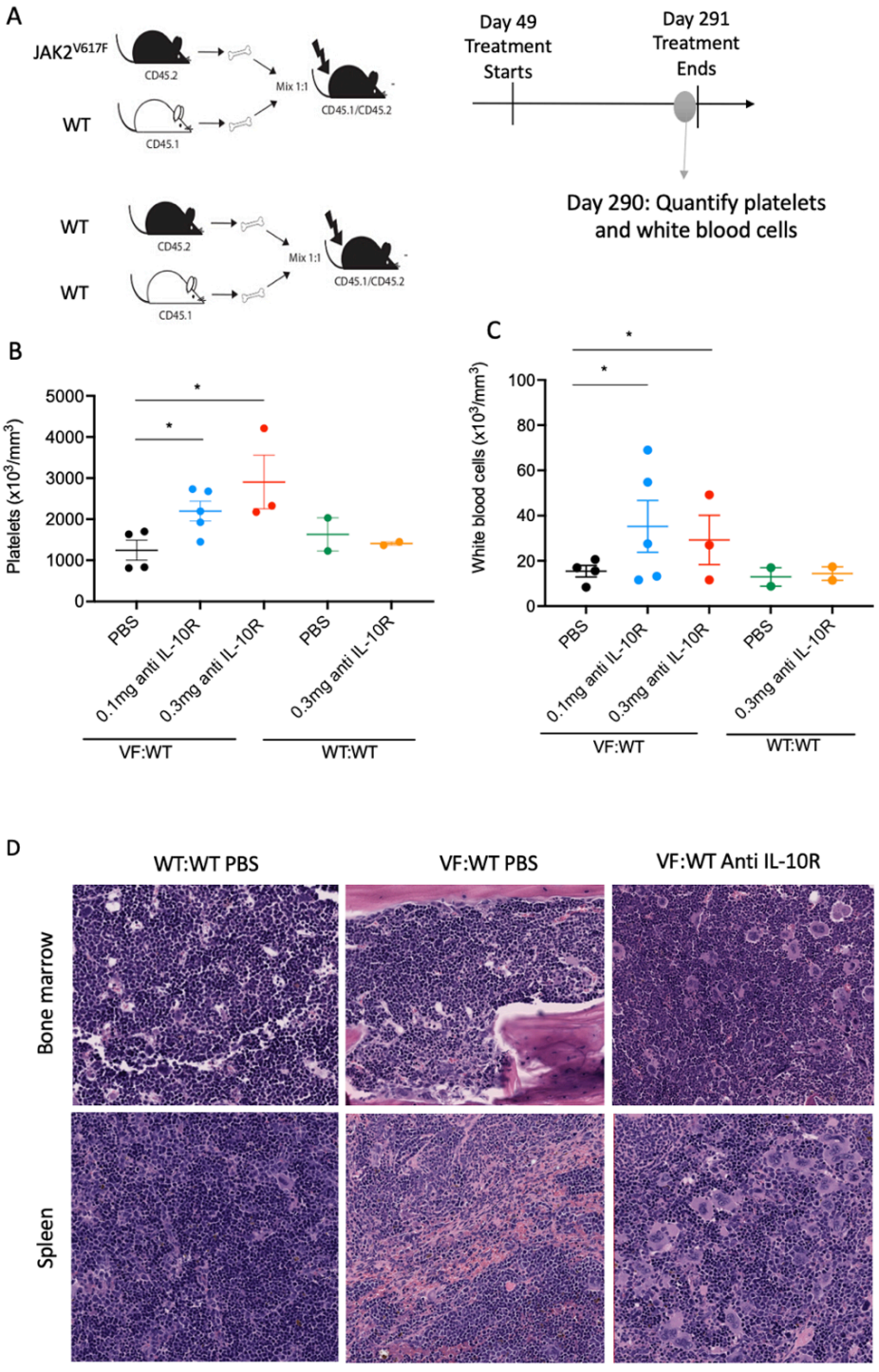


Fig 4.5. IL-10R blockade exacerbates MPN disease progression. Experimental design of competitive transplantation assay of 1:1 ratio of JAK2^{V617F} and WT whole bone marrow into irradiated recipients. 1:1 ratio of WT:WT whole bone marrow was also transplanted separately as control. Treatment of 0.1mg or 0.3mg of IL-10 blockade, and PBS control started on day 49 and ended on day 291. (B) Quantification of platelets for all groups on day 290 post transplantation. (C) Quantification of white blood cell count for all groups on day 290 post transplantation. (D) H&E histology of bone marrow and spleen post harvesting.

4.4 Discussion

We have previously identified blunted IL-10 signaling in MPN monocytes [21], which indicates that impairment in the IL-10 signaling pathway. IL-10 signaling is important for modulating immune response[22, 31, 32] and has increasingly been recognized to have a protective role in the self-renewal function of HSCs [29, 33-35]. Several studies have established that chronic inflammation, such as those induce by repeated exposure to inflammatory stimuli LPS, causes HSCs to cycle more than they normally do, which leads to loss self-renewal function and eventually reduced WT HSC population [25, 36]. This is concept is critical in understanding clonal expansion in MPN, because MPN arises from a single somatic mutation such as the $JAK2^{V617F}$.

We first investigate how an impairment in IL-10 signaling affects the fitness of HSCs. We first shown that blocking IL-10 signaling prolong the proliferation of HSCs in response to LPS stimulation, which suggests that IL-10 signaling is required for HSCs to stop cycling and return to homeostasis to prevent from stress induced from exhaustion. We also assessed HSC repopulation capability using a competitive transplantation assay to show that chorionic exposure to the combination of IL-10 signaling and LPS significantly accelerate the loss of HSC repopulation capacity compared to unmodified and untreated HSCs. These results indicate that blocking IL-10 signaling has negative impact on the fitness of HSCs and its ability to repopulate mature blood cells.

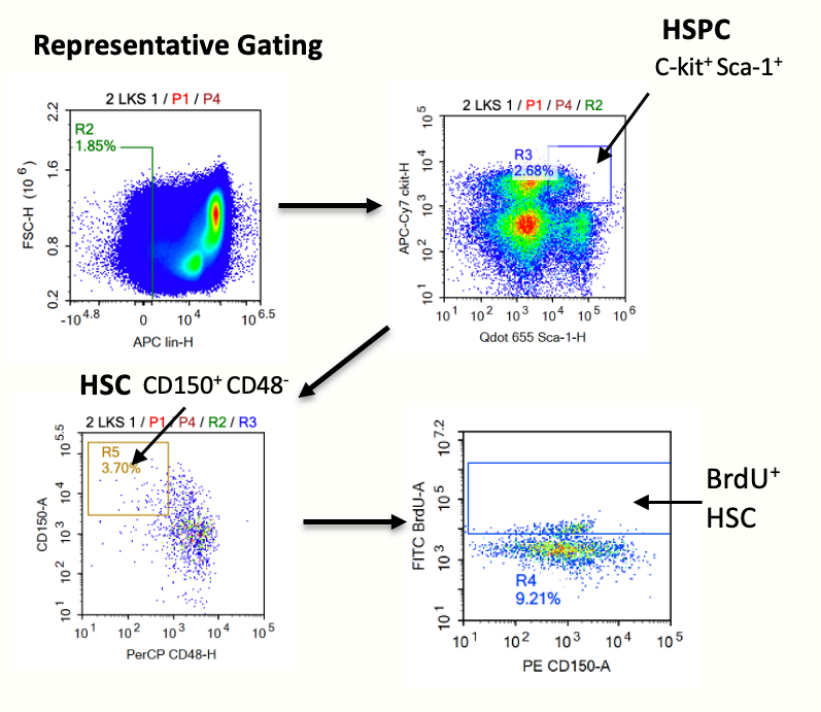
We next tested the hypothesis that IL-10 signaling blockade impair WT HSCs more than $JAK2^{V617F}$ HSCs. We found that blocking IL10 signaling have a greater impact on the proliferation of WT HSCs than $JAK2^{V617F}$ HSCs when exposed to LPS stimulation. This result

suggests that WT HSCs are more prone to exhaustion in the presence of IL-10 defects than JAK2^{V617F} HSCs. Our data from the competitive transplantation assay also demonstrated that chronically blocking IL-10 signaling provide a better selective advantage for *JAK2*^{V617F} HSCs to expand. We also found that blocking IL-10 signaling leads to enlarge spleen, increased platelets and white blood cells, and infuriation of megakaryocytes in the bone marrow and spleen that was not found in the control group. Together, these results indicate that IL-10 signaling blockade provides *JAK2*^{V617F} HSCs selective advantage to expand and exacerbate disease progression.

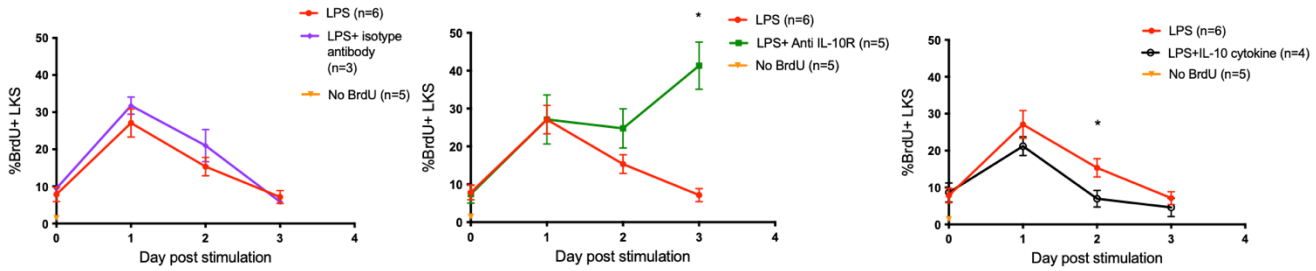
It is still unclear how IL-10 blockade has less negative impact on *JAK2*^{V617F} HSCs fitness than WT HSCs. We hypothesis that *JAK2*^{V617F} enhance the IL-10 signaling pathway, and thereby restoring the signal that is important for sustaining its self-renewal function. One possibility is that *JAK2*^{V617F} can directly interact with the IL-10R and restore the IL-10 signaling in MPN patients. Because WT *JAK2* is unable to interact with IL-10R, they are more impacted by blunted IL-10 signaling.

We are also interested in identifying potential genetic predisposition in IL-10R signaling defects. MPN has strong association with many autoimmune diseases such as ulcerative colitis, systemic lupus erythematosus, Crohn's disease, and autoimmune encephalomyelitis that have also been found to have dysregulation in the IL-10 signaling pathway [37-40]. A great interest in this area would be to identify the defects in IL-10 signaling that may be shared in patients with MPN and autoimmune diseases.

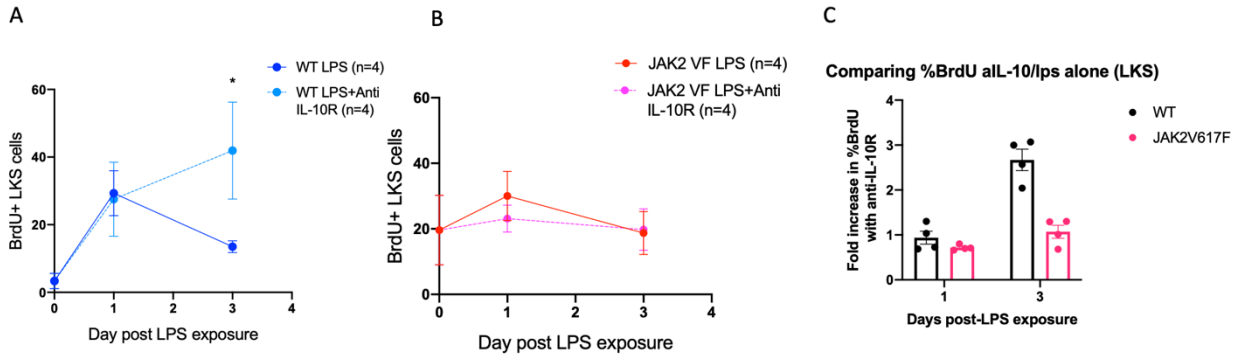
4.5 Supplementary Information



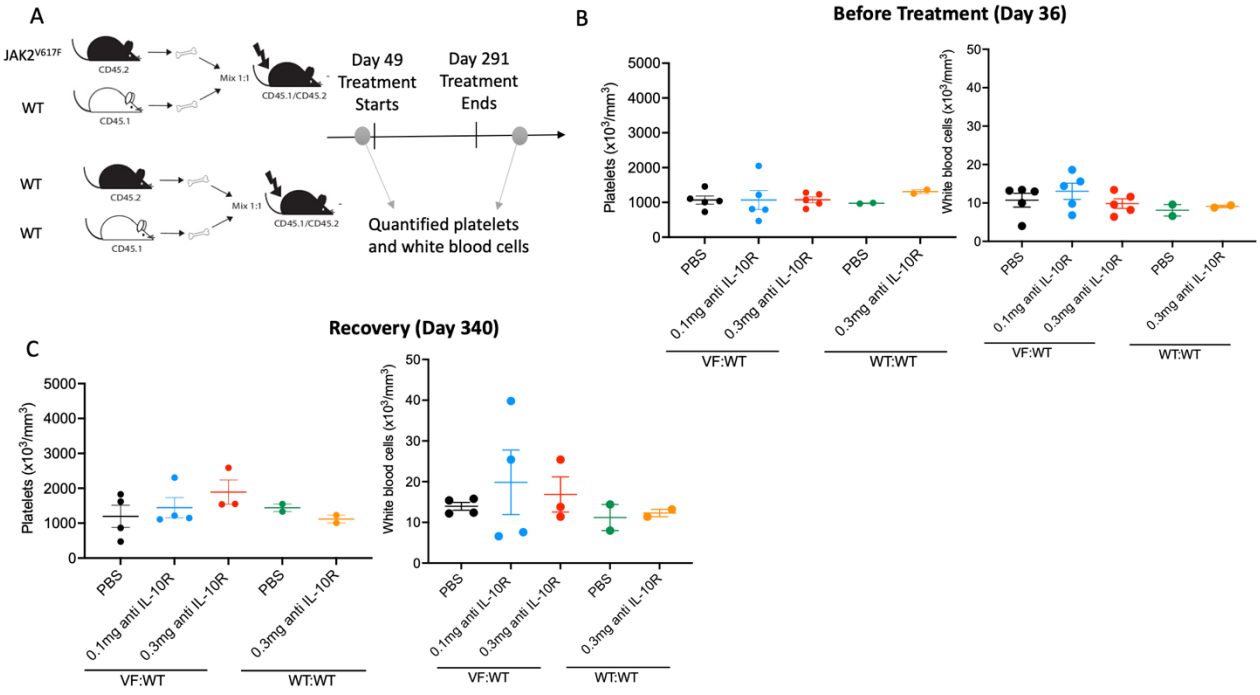
Supplemental Figure 4.1 Representative gating of BrdU+ HSPC and HSC.



Supplemental Figure 4.2 Percent of BrdU+ WT HSPCs were quantified by LKS markers for the group of LPS only and LPS+ anti IL-10R.



Supplemental Figure 4.3 Percent of BrdU^{+} *Jak2*^{V617F} and WT HSPCs were quantified by LKS markers for the group of LPS only and LPS+ anti IL-10R.



Supplemental Figure 4.4 Active IL-10R blockade is required to exacerbates MPN disease progression. (A) Experimental design of competitive transplantation assay of 1:1 ratio of JAK2^{V617F} and WT whole bone marrow into irradiated recipients. 1:1 ratio of WT:WT whole bone marrow was also transplanted separately as control. Treatment of 0.1mg or 0.3mg of IL-10 blockade, and PBS control started on day 49 and ended on day 291. (B) Quantification of platelets and white blood cells for all groups on day 36 post transplantation. (C) Quantification of platelets and white blood cells for all groups on day 340 post transplantation.

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Chapter 5

Overview and Future Directions

5.1 Overview

Myeloproliferative Neoplasm (MPN) is an excellent model disease to study the relationship between chronic inflammation and the emergence of mutant clones which lead to blood cancers. By understanding the underlying cause of inflammation that drives the pathogenesis of MPN, we could provide better therapeutic targets and prevention for disease development. Chronic inflammation is not only responsible for many of the symptoms in MPN [1-4], but also creates an environment which favors growth of mutant cells over wild-type (WT) cells.

Dr. Fleischman has previously demonstrated that the *JAK2*^{V617F} mutant hematopoietic progenitors are resistant to the negative effects of the inflammatory cytokine Tumor Necrosis Factor alpha (TNF-α) [5]. Here, we found that MPN monocytes persistently produce TNF-α after Toll-like-receptor (TLR) agonist stimulation due to defective negative feedback regulation of TLR signaling. MPN monocytes are less sensitive to IL-10 [6], a key anti-inflammatory cytokine whose role is to dampen TLR signaling [7, 8]. It is unknown whether this blunted IL-10 signaling in MPN occurs before or after the somatic acquisition of *JAK2*^{V617F}.

We first determined whether this aberrant cytokine production is exclusively found in *JAK2*^{V617F} cells. Our results demonstrated that both WT and *JAK2*^{V617F} monocytes in MPN patients produce exaggerated amounts of TNF-α, suggesting that JAK2^{V617F} does not drive inflammation in a cell-intrinsic manner. In addition, we tested the possibility that JAK2^{V617F} monocytes may secrete factors to alter normal monocytes to produce inflammatory cytokines. Our results indicate that *JAK2*^{V617F} monocytes do not induce normal monocytes to produce more TNF-α. To examine the direct effect of *JAK2*^{V617F} alone, we used bone marrow

derived macrophages from a *JAK2*^{V617F} knock-in murine model and found that *JAK2*^{V617F} macrophages do not produce persistent TNF-α in response to TLR agonist as we observed in human MPN patients. Interestingly, we have data from a pair of discordant identical twins demonstrating that abnormally elevated TNF-α and reduced response to IL-10 signaling are found in the unaffected twin of an MPN patient. Together, these data suggest that chronic inflammation may be an intrinsic feature of MPN patients and may play a role in the predisposition to acquire MPN. We reasoned that the IL-10 signaling defects may also be a predisposition that play a role in the clonal expansion of malignant clone in MPN.

Several studies have suggested that IL-10 directly and indirectly regulates HSC self-renewal, and that that loss of IL-10 signaling results in a reduction of the HSC population [7, 9-12]. We tested the hypothesis that IL-10R blockade impairs WT HSCs more than *JAK2*^{V617F} HSCs. Our results suggested that IL-10 blockade induced more WT HSCs to proliferate than compared to *JAK2*^{V617F} HSCs when exposed to inflammatory stimuli. Preforming a functional assay for WT and *JAK2*^{V617F} competitive transplantation, we have demonstrated that IL-10R blockade results in an expansion of *JAK2*^{V617F} HSCs. In addition, we found that IL-10R blockade results in an exacerbation of MPN disease phenotype, which features a significant increase in platelets and white blood cells, enlarged spleen, and the abnormal infiltration of megakaryocytes in bone marrow and spleen. Collectively, these results suggest that impairment in IL-10R signaling may be a predisposition in MPN and that it provides *JAK2* ^{V617F} HSC a selective advantage to outcompete wild-type HSC, which allow *JAK2* ^{V617F} to drives clonal expansion in MPN (figure 5.1).

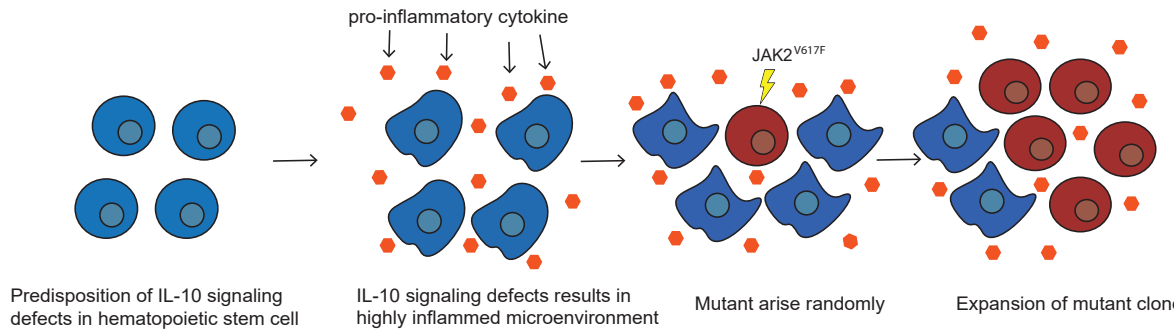

Figure 5.1 Working model of clonal expansion of hematopoietic malignancies in MPN patients with a predisposition of IL-10 signaling defects.

5.2 Future Directions

Our most recent findings suggest that IL-10 blockade reduces the fitness of the wild-type HSCs, but not the *JAK2*^{V617F} HSCs. We are interested in elucidating the molecular mechanism between *JAK2*^{V617F} and IL-10 receptor. One possibility is that *JAK2*^{V617F} directly interacts with IL-10R, which serves to restore the signaling of IL-10R in MPN cells. This may explain why IL-10R blockade does not have a significant impact on *JAK2*^{V617F} in cell cycling upon inflammatory stimulation. If IL-10R signaling is enhanced in *JAK2*^{V617F} HSCs, they would be able to accelerate return to quiescence after inflammatory signaling.

We propose to use Ba/F3 cells, an IL-3 dependent mouse cell line derived from the BALB/c mouse, to dissect cytokine receptor signaling pathways and to test the oncogenicity of mutations [13]. Parental Ba/F3 require IL-3 to provide a survival signal utilizing endogenous IL-3R [14]. Ectopic expression of activated tyrosine kinases or constitutively active cytokine receptors provide Ba/F3 cells with a survival signal which obviates the need for supplemental IL-3 [15, 16]. *JAK2*^{V617F} renders Ba/F3 cells cytokine independent (able to grow without supplemental IL-3) only when co-expressed with a cytokine receptor to which it binds and

activates is present, such as Thrombopoietin receptor (MPL) or Erythropoietin receptor (EPOR) [17]. Ba/F3 cells endogenously express IL-10Rα. We have created stable Ba/F3 cell lines ectopically co-expressing mIL-10Rβ (hCD4 tagged vector) and mJAK2^{V617F} (GFP tagged vector). To serve as controls we have created Ba/F3 cells expressing IL-10Rβ + WT JAK2 and BaF/3 cells expressing IL-10Rβ + empty GFP vector. Our preliminary data suggested that the Ba/F3 cells expressing IL10R *JAK2^{V617F}* proliferate at a higher those expressing IL10R MIG (figure 5.2A). This result suggests that *JAK2^{V617F}* enhances the IL-10 signaling pathway. Nitya Mehrotra, an undergraduate researcher in the Fleischman lab, is currently following up with this project to determine whether IL-10R and *JAK2^{V617F}*can interact to enhance the IL-10 signaling pathway (figure 5.2B)

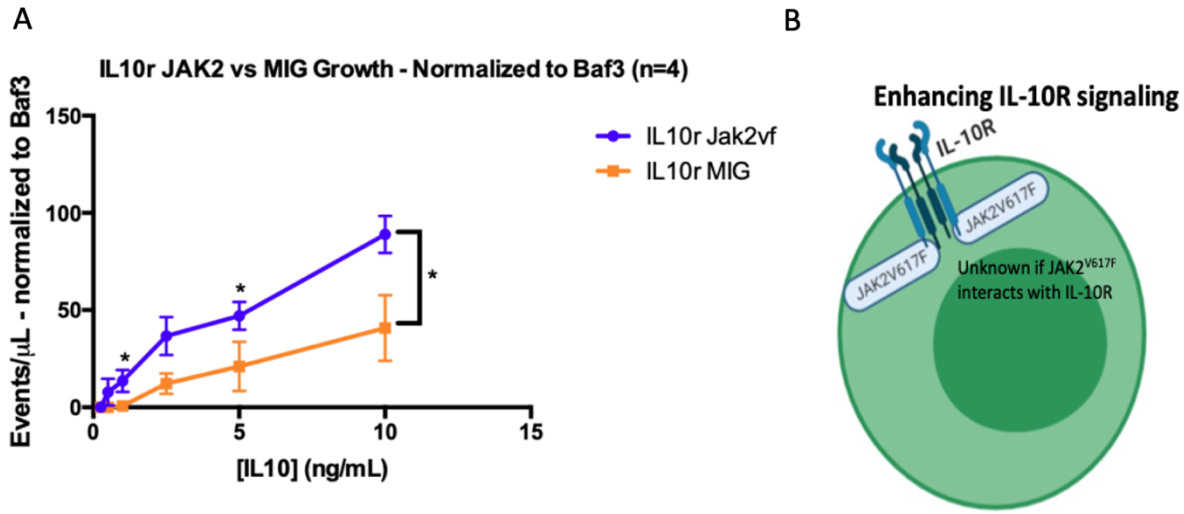

Figure 5.2 Using Ba/F3 cells to understand the signaling pathway of IL-10R and *JAK2^{V617F}*. (A) Ba/F3 cells count in event/µL for IL-10R *JAK2^{V617F}*and IL-10R MIG with the increase addition of IL-10 cytokine (B) Working model to determine whether *JAK2^{V617F}*interacts with IL-10R to enhance IL-10 signaling.

We are also interested in addressing whether defects in IL-10 signaling is a predisposition that occur prior the acquisition of *JAK2*^{V617F} mutation. We propose to examine T cells, the population that does not harbor *JAK2*^{V617F} mutation. If IL-10 signaling defects were found in T cells of MPN patients, then it would suggest that defects in IL-10 signaling may have occurred prior to acquiring the *JAK2*^{V617F} mutation (figure 5.3). We plan to specifically examine T cells differentiation from naïve T cells because IL-10 signaling suppresses Th17 differentiation. We will quantify Th17 differentiation with intracellular staining and ELISA for IL-17 [18], which is the cytokine that Th-17 exclusively produce and is used to identify Th-17 differentiation. If our hypothesis that IL-10 signaling is defective in T cells is supported, then we should observe that IL- 10 is less able to suppress Th17 differentiation in MPN compared to normal.

IL-10 is also important in suppressing Interferon gamma (IFN-γ) production in Th1 cells [19]. We propose to stimulate naïve T cells to differentiate into Th1, and then stimulate with exogenous IL-10 to measure IFN-γ using intracellular staining and ELISA. If IL-10 is less able to suppress IFN-γ in MPN patients than in normal controls, then this would indicate that T cells have signaling defects in the IL-10 cascade. Together, these experiments will allow us to determine whether or not the defects in IL-10 signaling are found in cells without *JAK2*^{V617F}, and if true would suggest that impairment in IL-10 signaling is a predisposition in MPN.

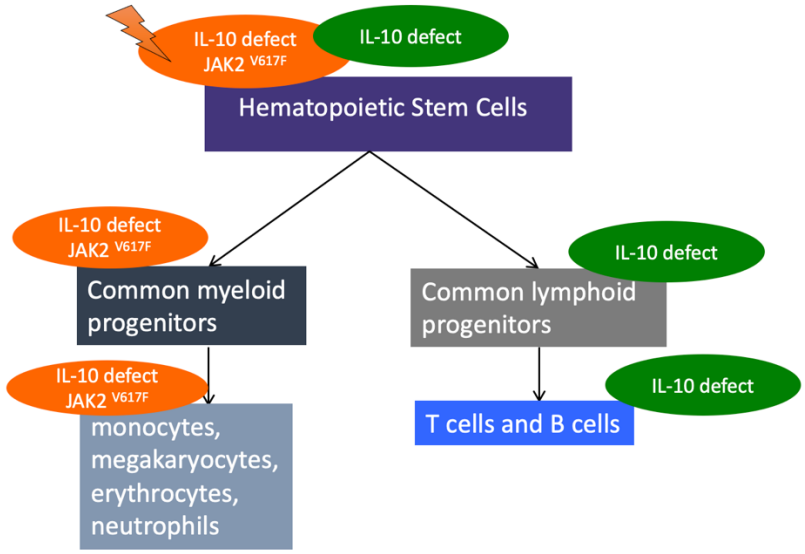

Figure 5.3 Working model for determining whether T cells, a population that does not harbor *JAK2*^{V617F} mutation, has impairment in IL-10 signaling.

Our last major future direction is to examine the familial MPN patients. There are emerging studies that suggests familial predisposition to acquire MPN [20, 21]. First degree relatives of MPN patients have a five-fold increased risk of developing MPN [22, 23]. In MPN families, HSCs which have acquired MPN driver mutations such as JAK2^{V617F} must have a strong selective advantage for these clones to independently emerge in multiple family members. We are interested in investigating whether there are association of single nucleotide polymorphisms (SNPs) in IL-10 pathway genes with affected Familial MPN patients compared to their unaffected family members. Genome wide associated studies (GWAS) have identified SNP such as in tyrosine kinase (TYK2) and other IL-10 gene to be associated with several autoimmune disease [24, 25].

MPN patients have chronic inflammation and are known to be associated with increased risk of autoimmune disease [26-29]. We propose to compare the germline SNPs of affected and unaffected Familial MPN members which would allow us to determine whether there are

germline predispositions that increase the risk of acquiring MPN. We hypothesize that familial MPN patients have associated SNPs in the IL-10 signaling pathway genes different from their unaffected family members.

We are currently collecting nail and blood samples from Familial MPN members. Affected MPN patient are those who harbor MPN initiating somatic mutation such as JAK2 ^{V617F} and CALR mutation. We will compare them to their unaffected family member, those who do not have these mutations. We will use whole exome sequencing to identify SNPs within the coding regions that are found in both blood and nail samples. Within the MPN family, we will compare the SNPs of affected and unaffected members. We will specifically search for SNPs in the IL-10 signaling pathway comparing the affected and unaffected MPN members within each family. This will allow us to determine whether IL-10 signaling defects is a result of germline predisposition that is associated with the risk of developing MPN.

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