UNIVERSITY OF CALIFORNIA,
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Investigation of the role of inflammation in myeloproliferative neoplasms

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Chemistry

by

Brianna Hoover

Dissertation Committee:
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CHAPTER 1 & 4

CHAPTER 2

In preparation for submission.

CHAPTER 3

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DEDICATION

I dedicate this work to my parents Laurie and Thomas Craver Jr.,

who bestowed their unconditional love and support

and instilled a passion for education in their children.

The 325,000 patients with cancer who are going to die this year cannot wait;
nor is it necessary, in order to make great progress in the cure of cancer,
for us to have the full solution of all the problems of basic research…
the history of Medicine is replete with examples of cures obtained years, decades,
and even centuries before the mechanism of action was understood for these cures.

Sidney Farber

The career I have chosen is laden with opportunity
yet it is fraught with heartbreak and despair
and the bodies of those who would have failed,
were they piled one atop another,
would cast a shadow down upon all the pyramids of the earth.

Yet I will not fail, as the others,
for in my hands I now hold the charts
which will guide me through perilous waters
to shores which only yesterday seemed but a dream.

Og Mandino
"The Greatest Salesman in the World"

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ABSTRACT OF THE DISSERTATION

Investigation of the role of inflammation in myeloproliferative neoplasms

By

Brianna Hoover

Doctor of Philosophy in Biological Chemistry

University of California, Irvine, 2021

Associate Professor Angela Fleischman, Chair

Myeloid malignancies occur when a hematopoietic stem cell (HSC) acquires a mutation that provide the stem cell clone with a competitive advantage over the remaining stem cell pool. Patients with myeloproliferative neoplasms (MPN) most commonly present with a gain-of-function mutation in Janus kinase 2 ($JAK2^{V617F}$), leading to constitutive JAK-STAT signaling activation and uncontrolled proliferation of myeloid cells. What remains unclear is how $JAK2^{V617F}$ provides HSCs with a competitive advantage over the non-mutant stem cell pool. Given that patients with MPN have chronic inflammation, we posit that inflammation plays a critical role in the onset and progression of disease. This thesis aims to identify how the JAK2 mutation influences response to inflammatory stress as well as competitive fitness in HSCs and impacts on disease pathology. To test how $JAK2^{V617F}$ HSCs respond to inflammation, we used transgenic knock-in mouse models to measure cell proliferation, oxidative stress and DNA damage using flow cytometry. While JAK2 mutant cells were hyperproliferative under homeostatic conditions, normal and mutant HSCs had similar proliferation levels following acute inflammation. To test the cell-intrinsic differences between stem cell responses, we performed competitive transplants and observed that the hyperproliferative phenotype of $JAK2^{V617F}$ stem cells was lost. This data

suggests that the bone marrow milieu plays an important role in stem cell activation and proliferation.

The second aim of this thesis is to therapeutically modulate inflammation and observe stem cell responses to inflammation as well as effects on disease pathologies. We found that the antioxidant agent n-acetylcysteine (NAC) extended the lifespan of transgenic knock-in mice with MPN presumably from inhibition of thrombosis. NAC had no effects on platelet activation but rather reduced platelet-leukocyte aggregation and neutrophil extracellular traps in knock-in mice. Taken together, NAC is an attractive therapeutic for MPN sequelae given its numerous effects as an anti-inflammatory and anti-thrombotic agent. An investigator initiated clinical trial is in development using NAC in MPN patients with an Investigational New Drug application pending at the FDA. Future research can also explore the combination of aspirin and NAC to reduce thrombotic risk, lower chronic inflammation, and possibly improve stem cell fitness in patients.

INTRODUCTION

Every day, the human body produces ~500 billion blood cells under highly controlled mechanisms to maintain a precise balance in the bloodstream (1). The balance of hematopoiesis can be thrown off, however, upon the acquisition of certain mutations in blood-forming stem cells, or hematopoietic stem cells. Hematopoietic stem cells are responsible for responding to local cues within the bone marrow following hematopoietic stress such as infection or major blood loss. Under homeostatic conditions, these blood-forming stem cells remain quiescent in order to maintain the cell fitness throughout the lifespan of the individual. When a stem cell exits quiescence and initiates cycling, mutations in the DNA can occur due to faulty repair mechanisms causing proliferation-induced stress (2,3). Most mutations that occur are unimportant and have no impact on the function of stem cells. Rarely, a mutation occurs in an important gene that improves the stem cell's ability to compete with the remaining pool of cells. This is the phenomenon of clonal hematopoiesis (CH), where a stem cell clone gains a selective advantage over remaining stem cells and eventually presents with a detectable population of mutant cells in the bloodstream (typically 2% of total blood cells). What has been surprising about clonal hematopoiesis is that it is markedly more prevalent in the population than leukemia is, where CH occurs in ~10% of individuals over 70 while the risk of developing leukemia occurs in only ~1% of individuals over 70 (4,5). In recent years, the improvements on sequencing technologies have greatly increased the ability for clinicians and researchers to detect CH in patients and to gain insights on the factors that influence hematopoietic stem cell competitive advantage.

Our lab studies a unique mutation that is present in ~3% of CH patients but is responsible for driving ~90% of myeloproliferative neoplasm (MPN) cases (6-8). MPN is characterized by the

overproduction of differentiated myeloid cells and is most commonly caused by an activating mutation in the Janus kinase 2 (JAK2), called $JAK2^{V617F}$. This valine to phenylalanine mutation induces a conformational change in JAK2 and leads to constitutive activation of JAK-STAT signaling in the mutant cells and their progeny. JAK-STAT is a critical signaling pathway of the immune system and is activated by numerous cytokines and growth factors. JAK-STAT influences many other signaling pathways such as PI3K/AKT/mTOR, NF-κB and p38MAPK; thus, the $JAK2^{V617F}$ mutation can cause a host of issues in an individual. The type of cell population that is overproduced characterizes the MPN diagnosis; for instance, polycythemia vera (PV) describes aberrant production of red blood cells, essential thrombocythemia (ET) describes aberrant production of platelets, and primary myelofibrosis (PMF) describes the development of fibrosis in the bone marrow, splenomegaly and subsequent cytopenia.

Since the discovery of this JAK2 mutation in 2005, much of the field has been focused on identifying how the mutation influences hematopoiesis but more importantly on how to selectively target and eliminate these cells (7-11). Obviously, JAK inhibitors were one of the first therapies tested to treat MPN and the JAK inhibitors Ruxolitinib and Fedratinib currently serve to reduce spleen size and minimize symptoms. However, given the importance of JAK signaling for normal hematopoiesis, patients on JAK inhibitors can exhibit dose-related cytopenias and even resistance after 2-3 years of use (12). Given the chronic nature of MPN, patients can survive for decades without exhibiting disease progression. Many MPN patients have a “watch and wait” management strategy though common therapies including hydroxyurea, anagrelide, and aspirin serve to reduce blood clotting risk (13). For high-risk patients, interferon-alpha is prescribed to reduce blood clotting risk and can reduce JAK2 mutant cell burden in some people (13). However, other than

potentially interferon-alpha, these treatments do not aim to target the root of the problem, which lies in the mutant stem cell pool.

Currently, the only way to "cure" MPN is by allogeneic hematopoietic cell transplant. Not only is it difficult to find bone marrow donors that match the patient, but the transplant has complications such as graft failure or graft versus host disease, leading to high mortality in patients (14). The ideal therapeutic would selectively target the JAK2 mutant stem cells and their progeny without stressing the normal hematopoietic system. Thus, our approach is to identify winning strategies that JAK2 mutant hematopoietic stem cells use to outcompete normal cells. **The ultimate goal of our lab is to identify how JAK2 mutant stem cells gain a competitive advantage and to develop novel therapies to selectively target these mutant stem cells.**

What remains puzzling is that constitutive activation of JAK-STAT drives proliferation of stem cells and should exhaust these stem cells. In normal stem cells, fitness is harmed by proliferative stress induced by exiting quiescence and gaining DNA mutations during replication, leading to a reduced ability to engraft in an irradiated host (15). Thus, stem cell proliferation and competitive fitness are inversely related. Studies on other CH mutations have identified common winning strategies that mutant HSCs utilize to gain a competitive advantage, and these include reduced apoptosis or increased survival to exogenous stress or enhanced self-renewal compared to the stem cell pool (6). Chronic inflammation is a driver of stem cell proliferation and exhaustion and the importance of inflammation in MPN is discussed at length in Chapter 1. In Chapter 2, we test the hypothesis that constitutive activation of JAK-STAT signaling in mutant HSCs renders cells resistant to inflammatory stress by focusing on the stem cell responses to inflammation in a transgenic knock-in mouse model of MPN. Normal stem cells are more quiescent at rest and may therefore be more readily available to respond to inflammation. Specifically, we investigate the

interplay of inflammation, oxidative stress, proliferation and DNA damage following acute and chronic treatment with the bacterial component lipopolysaccharide. Some key questions we explore in Chapter 2 include: Do JAK2 mutant HSCs have a dampened response to LPS-induced inflammation by measure of proliferation? Does the presence of JAK2 mutant cells influence the normal bystander cells response to inflammation? Can stem cell responses and/or competitive fitness be spared by anti-inflammatory or antioxidant drugs?

In chapters 3 and 4, we explore the impact of therapeutics on disease complications and pathologies. Chapter 3 explores the unexpected finding that the antioxidant N-acetylcysteine (NAC) extends the lifespan of a knock-in mouse model of MPN. These mice die suddenly ~3 months of age presumably from thrombotic events, but NAC treatment extended the average lifespan of these mice to ~12 months. Furthermore, mice treated with NAC produced fewer pulmonary thrombi and increased survival following a chemically induced in vivo thrombosis assay. We performed biochemical assays to show that the antithrombotic effect is not by inhibition of platelet activation but rather by reduction of platelet-leukocyte interactions and reduced neutrophil extracellular traps. Finally, in Chapter 4 we aimed to therapeutically target JAK2 mutant cells based on our knowledge of dysregulated apoptosis pathways in leukemic cells. Many cancer cells evade apoptosis by upregulating inhibitor of apoptosis proteins and this phenomenon can be exploited using mimetics of second mitochondria-derived activator of caspases (SMAC) to induce apoptosis in these cells. Indeed, we find that $JAK2^{V617F}$ drives the overexpression of inhibitor of apoptosis proteins, yielding these cells sensitive to apoptosis in the presence of a SMAC mimetic but only in the absence of TNF-driven inflammation. A better understanding of how JAK2 mutations influence stem cell biology, leukocyte interactions and pathologies is critical for improving MPN therapies of tomorrow.

Key Abbreviations

HSC – hematopoietic stem cell
HSPC - hematopoietic stem and progenitor cell
CH (or CHIP) – clonal hematopoiesis (of indeterminate potential)
JAK – Janus kinase
STAT - Signal Transducer and Activator of Transcription proteins
MPN – myeloproliferative neoplasm
PV – polycythemia vera, a subtype of MPN characterized by too many red blood cells
ET – essential thrombocythemia, a subtype of MPN characterized by too many platelets
PMF – primary myelofibrosis, a subtype of MPN characterized by fibrosis in BM
MDS – myelodysplastic syndrome
TNFα – tumor necrosis factor alpha
BM – bone marrow
BMDM – bone marrow-derived macrophages
PB – peripheral blood
CBC – complete blood count
WBC – white blood cell
WT – wildtype
$JAK2^{V617F}$ (or VF) – the most common mutation that causes MPN by converting valine into phenylalanine at the 617^{th} amino acid of Janus kinase 2
VF-KI - $JAK2V617F^{fl/fl}$:*Vav1*-Cre knock-in mouse model
LPS – lipopolysaccharide
ELISA – enzyme linked immunosorbent assay
ROS – reactive oxygen species
FACS – fluorescence-activated cell sorting
LKS – cell staining scheme to identify HSPCs ($Lineage^{-}ckit^{+}Sca1^{+}$)
LKS-SLAM – cell staining scheme to identify HSCs ($Lineage^{-}ckit^{+}Sca1^{+}CD150^{+}CD48^{-}$)
MPP2/3 – cell staining scheme to identify multipotent progenitors ($Lineage^{-}ckit^{+}Sca1^{+}CD150^{-}CD48^{+}$)
NAC – N-acetyl-l-cysteine
PLA – platelet leukocyte aggregate
NET – neutrophil extracellular trap
SMAC – second mitochondria-derived activator of caspases
CALR – calreticulin
IAP – inhibitor of apoptosis protein
NF-κB – nuclear factor kappa light chain enhancer of activated B cells
ERK – extracellular signal-related kinase

CHAPTER 1

Review

The Critical Role of Inflammation in the Pathogenesis and Progression of Myeloid Malignancies

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Abstract: Hematopoietic stem cells (HSCs) maintain an organism's immune system for a lifetime, and derangements in HSC proliferation and differentiation result in hematologic malignancies. Chronic inflammation plays a contributory if not causal role in HSC dysfunction. Inflammation induces HSC exhaustion, which promotes the emergence of mutant clones that may be resistant to an inflammatory microenvironment; this likely promotes the onset of a myeloid hematologic malignancy. Inflammatory cytokines are characteristically high in patients with myeloid malignancies and are linked to disease initiation, symptom burden, disease progression, and worsened prognostic survival. This review will cover our current understanding of the role of inflammation in the initiation, progression, and complications of myeloid hematologic malignancies, drawing from clinical studies as well as murine models. We will also highlight inflammation as a therapeutic target in hematologic malignancies.

Keywords: myeloid malignancy; clonal hematopoiesis; hematopoietic stem cells; symptom burden; inflammation

1.1. Introduction

Inflammation drives tumor progression in multiple cancer types [1]. While the role of inflammation in the initiation and progression of many solid tumors is well established, our understanding of how inflammation promotes initiation and progression of hematologic malignancies is an emerging field. Hematologic malignancies arise from the expansion of mutated hematopoietic stem cell (HSC) clones with uncontrolled proliferation or differentiation. Myeloid hematologic malignancies can manifest with a wide range of phenotypes. Acute myeloid leukemia (AML) is characterized by a blockade in differentiation with explosive expansion of immature hematopoietic cells (blasts) that is rapidly fatal without treatment. In myeloid diseases with a more chronic course, such as myeloproliferative neoplasms (MPN) and chronic myelogenous leukemia (CML), differentiation is intact but there is aberrant expansion of mature myeloid cells. In myelodysplastic syndrome (MDS), hematopoietic precursors are expanded in the bone marrow, but there is ineffective hematopoiesis leading to low peripheral blood counts. In order for a myeloid malignancy to arise, the mutated HSC must outcompete the non-mutated competitor HSCs. Therefore, the selective pressures imposed upon an organism's HSC pool define which mutant clones will have a selective advantage and expand at the expense of their normal counterparts. Chronic inflammation is a common selective pressure that leads to impaired fitness of normal HSCs. In this review, we will highlight how inflammation drives the emergence of mutant HSC clones, promotes myeloid malignancy progression, and exacerbates symptom burden. A clear understanding of how inflammation drives each of these processes is key to developing interventions that prevent disease initiation, halt progression, and improve quality of life in myeloid malignancies.

1.2. Inflammation Is a Clinical Concern for Myeloid Malignancies

1.2.1. Chronic Inflammation Is a Shared Characteristic in Myeloid Malignancies

A shared characteristic of many hematologic malignancies is the overproduction of inflammatory cytokines. Although several cytokines are overexpressed in myeloid malignancies, overproduction of tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) is most commonly observed in patients, suggesting that these cytokines play a role in the fundamental aspects of the development and/or manifestations of hematologic malignancies (Figure 1.1). Some cytokines are uniquely elevated in specific myeloid malignancies, suggesting that these cytokines may have a specific role in the pathogenesis of the hematologic malignancy in which they are overexpressed. In myeloid malignancies, the anti-inflammatory cytokines, IL-4, IL-10 and IL-13 are also commonly elevated in patients (Figure 1.1), likely as a response to the chronic inflammatory state in an attempt to dampen inflammation.

Elevated cytokines are commonly linked to a worsened prognosis, particularly in MPN, as indicated by the bolded biomarkers in Figure 1.1. In treatment-naive MPN patients, increased levels of IL-8, IL-2R, IL-12, IL-15, and IP-10 were independently predictive of inferior survival [2]. A normal age-related phenomenon is that inflammatory cytokines increase with age (referred to as inflamm-aging). Aging-induced inflammation likely plays a role in initiation as well as progression of myeloid malignancies. One study compared plasma cytokine levels in AML patients that were younger or older than 65 years of age versus aged-matched normal controls. In agreement with previous literature, IL-6 and IL-8 were significantly elevated in older healthy patients compared to younger healthy patients [3]. However, AML patients with elevated IL-6 and IL-17A had an even worse prognostic survival compared to AML patients with only high IL-6. This finding suggests that cytokines play synergistic roles**,** which may in part explain why prognostic survival is correlated with multiple elevated cytokines rather than just a single elevated

cytokine in AML and other malignancies [3]. Further, AML patients with elevated IL-10 and decreased IL-6 were correlated with improved patient survival, demonstrating the importance of maintaining a balance between pro- and anti-inflammatory cytokines [3].

Inflammatory cytokines can be used as a predictor of patient response to therapies. Nievergall and colleagues reported that newly diagnosed CML patients exhibited several elevated inflammatory cytokines in plasma compared to healthy controls [4] (Figure 1.1). Following three months of treatment with imatinib, a BCR-ABL kinase inhibitor, cytokine profiles from patients that were early molecular responders (EMR) were compared to patients that failed to achieve EMR. Strikingly, transforming growth factor alpha (TGFα) was uniquely elevated in the CML patients that failed EMR and was a more significant predictor of failed response to imatinib treatment than IL-6. While TGFα has long been investigated and linked to prognostic survival in many solid tumor types, this is the first evidence that TGFα is an important cytokine in a hematologic malignancy [4].

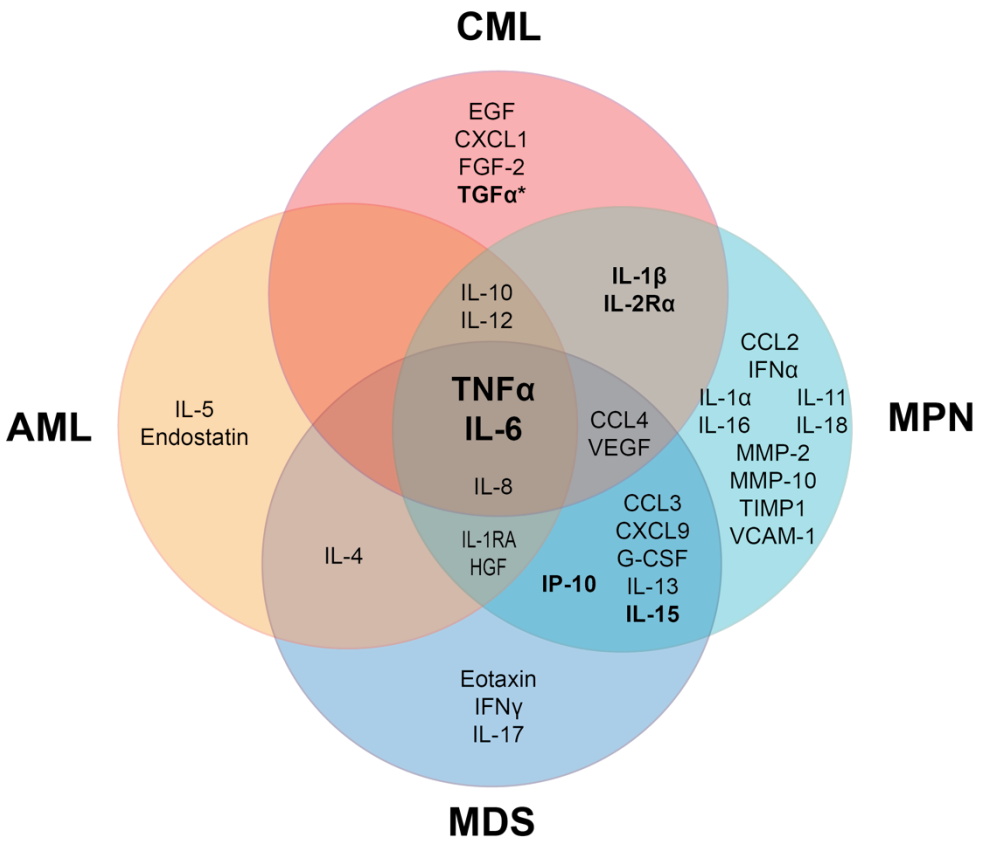

Figure 1.1. Common upregulated pro- and anti-inflammatory cytokines in patient blood plasma or serum compared to healthy controls. Elevated tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) are shared among the major myeloid malignancies. Cytokines in bold have been linked to prognostic survival in at least one hematopoietic malignancy. *Prognostic survival is correlated with transforming growth factor alpha (TGFα) only when IL-6 is also elevated. AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm. Referenced in [2–9]. EGF: epidermal growth factor; CXCL: chemokine (C-X-C motif) ligand; FGF: fibroblast growth factor; IL-1RA: interleukin 1 receptor antagonist; HGF: hepatocyte growth factor; IFNα: interferon alpha; CCL: chemokine (C-C motif) ligand; VEGF: vascular endothelial growth factor; G-CSF: granulocyte colony-stimulating factor; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinases; VCAM: vascular cell adhesion molecule.

1.2.2. Autoinflammatory and Autoimmune Diseases in Hematological Malignancies

Many studies have demonstrated an association between cancer and autoimmune diseases (AID). Elevated pro-inflammatory cytokines are found in the serum of patients diagnosed with AID. These cytokines likely play a critical role in the pathogenesis of AID and may also contribute to autoimmune-associated tumorigenesis [10]. Although the majority of studies on cancer and autoimmunity focus on solid tumors, some studies have highlighted a specific association with

hematologic malignancies. For example, specific autoimmune disorders such as systemic lupus erythematosus or autoimmune hemolytic anemia display a major risk of Non-Hodgkin Lymphoma [11]. Moreover, a chronic immune stimulation has been considered as a possible trigger for hematologic malignancies such as MDS and AML, which were increased significantly in patients with a broad range of autoimmune conditions [12,13]. Specifically, AML was most closely associated with systemic lupus erythematosus, rheumatoid arthritis, polymyalgia rheumatica, and autoimmune hemolytic anemia while MDS was associated with pernicious anemia and rheumatoid arthritis [12].

There is a growing body of evidence linking a genetic predisposition to chronic inflammation with MPN [14]. MPN patients and their unaffected family members have an increased incidence of autoimmune diseases [15,16]. Genome-wide association studies (GWAS) of MPN [17–19] and inflammatory diseases have identified associations with the same genes. For example, the Janus kinase 2 (JAK2) single nucleotide polymorphism (SNP) rs10758669, a SNP that tags the 46/1 haplotype associated with $JAK2^{V617F}$-mutated MPN, was also identified previously to be associated with Crohn's disease [20]. It has been proposed that the JAK2 46/1 haplotype results in an augmented response to cytokine stimulation, leading to increased inflammation [21]. Interestingly, a study found $JAK2^{V617F}$ in about 20% of people with mild thrombocytosis in an inflammatory bowel disease (IBD) clinic, suggesting that a significant fraction of people with IBD harbor a $JAK2^{V617F}$ clone [22]. In addition, MPN patients have a >2-fold increased risk of IBD, a result that links these two disease entities with potentially common predispositions. Moreover, SNPs in SH2B3 (Lnk) are associated with MPN [17], multiple sclerosis and atherosclerosis [23], though atherosclerosis is not an autoimmune disease but a chronic inflammatory state.

Autoinflammatory diseases are characterized by recurrent inflammatory episodes without any evidence of auto-antibodies, making them distinct from the autoimmune conditions. These diseases include Familial Mediterranean Fever (FMF), periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome (PFAPA), or adult Still's disease [24]. As these clinical entities are rare and less studied than AID, very few studies have investigated the incidence of cancer in these populations with a recurring inflammatory environment. Interestingly, a recent study on Israeli FMF patients showed a decreased cancer incidence compared to the general population [25]. Given that FMF is a chronic inflammatory state, one would expect that patients with FMF would have higher rather than lower cancer incidence. Further studies must be performed using a larger population of FMF patients to resolve these uncertainties. If FMF patients truly have a decreased incidence of cancer compared to the populous then it may be worth identifying whether particular circulating cytokines are responsible for this protective effect.

The relationship between hematologic malignancies and AID is bidirectional, as lymphoid and myeloid neoplasms classically present autoimmune manifestations, sometimes even revealing them. AID can also be triggered by treatments for hematologic malignancies; many chemotherapies and immune agents have been implicated in the development of autoimmune cytopenias [26]. Some hematological diseases can also be uniquely driven by an autoimmune pathogenesis. Primary autoimmune myelofibrosis has a benign course and is distinct from a neoplastic process, as seen in primary myelofibrosis or secondary to chronic myeloproliferative neoplasm [27]. The key difference between autoimmune myelofibrosis and MPN myelofibrosis is that a mutated HSC clone drives MPN myelofibrosis whereas in autoimmune myelofibrosis there is no evidence of a neoplastic clone.

1.2.3. Inflammation Exacerbates Symptom Burden in Myeloid Malignancies

Chronic inflammation worsens cancer-related symptoms [28]. Elevated proinflammatory cytokines such as TNFα, IL-6 and IL-1 are correlated with sickness behavior which consists of lethargy, depression, anorexia, anhedonia, cognitive impairment, hyperalgesia, and decreased social interaction [29]. This behavior has developed as an adaptive reaction during times of acute infection, but when these cytokines are chronically elevated in the context of cancer it has a significantly negative impact on quality of life. The impact of chronic inflammation on disease burden is particularly obvious in chronic MPNs which include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). MPNs most commonly result from somatic mutations in HSCs in JAK2, calreticulin, or MPL, with the central theme being constitutive activation of thrombopoietin receptor (MPL) and downstream activation of the Janus kinases and Signal Transducer and Activator of Transcription proteins (JAK/STAT) signaling pathway. In MPN patients, a plethora of inflammatory cytokines is elevated (demonstrated in Figure 1.1) and drives many of the debilitating symptoms associated with the disease [30,31].

The impact that physical symptoms have on quality of life and general well-being in patients with hematologic malignancies is under-recognized, as most therapies are focused on blood counts rather than symptoms. Recent studies have revealed how much the physical symptom burden weighs on the psychology of patients with hematologic disorders [32], and how this diagnosis can lead to distress, depression, and anxiety [33]. In aggressive hematologic malignancies such as AML, the major focus is on eradication of the leukemic clone because the consequence of not targeting the mutant clone is fatal. On the other hand, for chronic hematologic malignancies such as MPN, only a few MPN patients will inevitably fail treatments and see their disease progress rapidly or transform into acute leukemia. Because MPN is a chronic disease, the majority of MPN patients live with this diagnosis with ‘watch and wait’ management. However, this approach is

being challenged by the renaissance of interferon-alpha (IFNα) [34,35]. IFNα depletes MPN disease-initiating cells by inducing cell cycle activation of mutant HSCs and driving them to erythroid-lineage differentiation [36]. IFNα has been used for decades, even in low-risk patients, and can lead to molecular responses in a both JAK2 and CALR mutated MPN patients; moreover, these responses can be maintained for several years after discontinuation of IFNα [37–40]. In addition, IFNα therapy can blunt or even reverse fibrosis in early-stage MF patients [41]. The ability to modify disease course is unique to IFNα, in particular, at the early stages of disease, however its side effect profile poses a challenge to more widespread use. Ropeginterferon, a long-acting IFNα with a tolerable toxicity profile, is currently being evaluated in clinical trials [42]. The availability of an easily tolerated IFNα therapy may lead to more widespread use of IFNα in early disease MPN patients. Regardless, given that symptom burden has a major impact on the quality of life of MPN patients, there should be a focus on identifying therapeutics for managing symptoms in addition to targeting malignant HSC clones.

MPN patients present a broad-spectrum of symptoms, even in early-stage disease, and vary among individuals, though some symptoms are more prevalent in ET, PV, or PMF [43]. Regardless of the subtype, the most common complaint is fatigue (80.7%), followed by pruritus (52.2%), night sweats (49.2%), bone pain (43.9%), and fever (13.7%) [30]. Interestingly, specific symptoms and complaints have been correlated with elevated levels of specific inflammatory biomarkers, as shown in Figure 1.2. Other symptoms such as abdominal discomfort, early satiety, and numbness/tingling in the extremities are also frequent complaints in MPN [30]. The recently developed National Comprehensive Cancer Center Network (NCCN) guidelines for PMF address the importance of symptom burden and recommend intervention to reduce symptom burden regardless of the prognosis scoring category. Symptom scoring tools such as the Myelofibrosis

Symptom Assessment Form (MF-SAF) and the Myeloproliferative Neoplasm Symptoms Assessment Form (MPN-SAF) were developed in order to assess the clinical spectrum of physical symptoms that could affect the quality of life of MPN patients [44,45]. These tools were also used to objectively measure the impact of the JAK1/2-inhibitor, ruxolitinib, on symptom burden in the COMFORT-I trial [46]. Ruxolitinib, a potent anti-inflammatory drug, gained Food and Drug Administration (FDA) approval based on its ability to reduce spleen size and reduce symptom burden, which further highlights the central importance of symptoms in this disease. Treatment with Ruxolitinib resulted in reduction of multiple cytokines, including C-reactive protein (CRP), IL-1RA, macrophage inflammatory protein 1β (CCL4), TNFα, and IL-6 in PMF patients coincident with symptom improvement, further providing a link between inflammatory cytokines and symptom burden in MPN [9] (Figure 1.2).

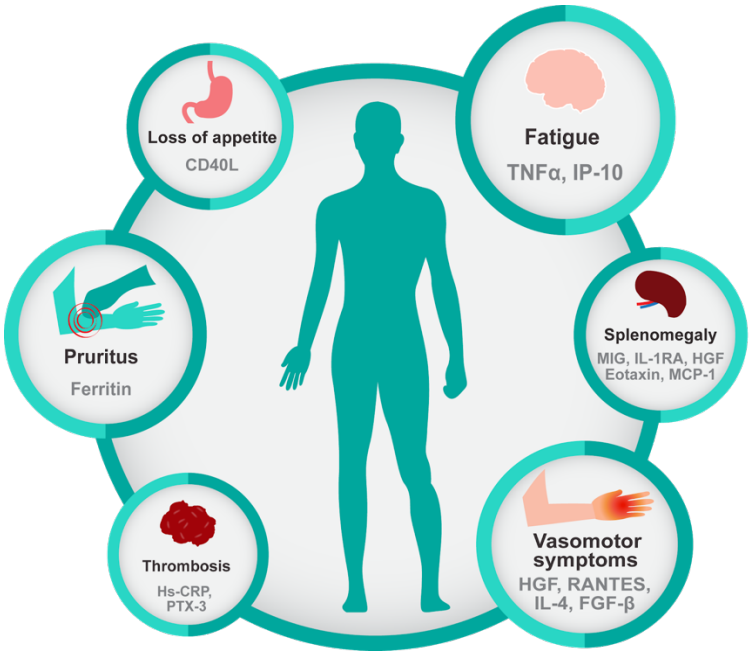

Figure 1.2. Inflammatory cytokines are correlated with characteristic features of myeloid malignancies. Common symptoms of myeloid malignancies, especially of primary myelofibrosis, include fatigue, pruritus, loss of appetite, and vasomotor symptoms and are significantly correlated with inflammatory biomarkers. Splenomegaly, which is inherent to the biology of MPN is also associated with increased inflammatory cytokines as is thrombosis a common complication of MPN. Referenced in [30,47,48].

1.2.4. Inflammatory Diseases and Cancers Share Therapies

The JAK/STAT signaling pathway plays a central role in hematopoiesis and the immune response, since constitutive activation of JAK/STAT signaling by gain-of-function mutations leads to the development of hematologic malignancies [49]. The discovery of the mutation $JAK2^{V617F}$ in 2005 has revolutionized our understanding of MPN diseases and allowed the creation of new targeted therapies to the Janus kinase (JAK) [50–54]. JAK inhibitors are also useful in autoimmune disease such as alopecia areata [55], psoriasis [56] and graft versus host disease [57,58]. Tofacitinib, a JAK2/JAK3 inhibitor, is FDA-approved for methotrexate-resistant forms of rheumatoid arthritis and psoriatic arthritis. The efficacy of JAK inhibitors is currently being investigated on solid tumors such as pancreatic, lung, colon and breast cancers [59] as well as inflammatory bowel diseases [60,61].

One potential explanation for the observed "double duty" of drugs for hematologic malignancies and autoimmune disease is shared signaling pathways that activate immune signaling and also drive leukemogenesis. However, the fact that anti-inflammatory drugs work well in hematologic malignancies also implies that inflammation mediated by host normal immune cells could facilitate disease progression, and that the true utility of the drug is not by blocking activation of signaling in the neoplastic cell, but through blocking inflammatory signals in non-neoplastic immune cells. A prime example is the use of ruxolitinib in MPN. A recent clinical trial investigating a JAK1 inhibitor, itacitinib, in myelofibrosis demonstrated that this agent had similar efficacy to Ruxolitinib [62]. This shows that a drug devoid of JAK2 inhibition but that has the ability to reduce inflammatory cytokines through JAK1 inhibition has a similar utility as a JAK2 inhibitor in MPN. In addition, a small study with etanercept, a TNFα inhibitor, demonstrated an

improvement in constitutional symptoms in myelofibrosis and showed only mild toxicity [63]. This suggests that much of the pathology in MPN may be driven by inflammation rather than the expansion of the neoplastic clone itself. Combining therapies that reduce inflammation and target the malignant clone is a rational approach towards a cure for MPN. Indeed, trials combining ruxolitinib and IFNα, which functions as an anti-inflammatory agent and specifically targets mutant cells, respectively, are underway and hold promise [64,65].

1.3. Inflammation Drives the Onset/Progression of Myeloid Malignancies

1.3.1. Inflammation Promotes Clonal Hematopoiesis

While substantial research has correlated chronic inflammation with myeloid malignancies, increasing evidence suggests that chronic inflammation may promote clonal hematopoiesis. Clonal hematopoiesis of indeterminate potential (CHIP), or simply clonal hematopoiesis (CH), is defined by the presence of cells in the blood or bone marrow with somatic mutations one or more of the 74 known mutations identified in hematological cancers. However, patients with CH have no overt phenotype of a hematological malignancy. Clonal hematopoiesis is an age-related condition and is correlated with increased incidence of non-hematological cancers [66] and atherosclerosis [67]. Clonal hematopoiesis can be found in over 10% of the population over the age of 70 and increases in prevalence with age [68]. Additionally, modern theories have attributed CHIP to being a significant cause of mortality and morbidity in developed countries, due to the pro-thrombotic nature of the disease course contributing to stroke and cardiovascular disease [67].

Inflammation likely plays a contributory if not causal role in hastening the onset of CHIP, though the mechanism linking inflammation to CHIP still remains uncertain. The model of the pathogenesis of CHIP in normal aging conditions and in a chronic inflammatory environment is

shown in Figure 1.3. Upon normal aging, a person will encounter multiple pathogens and will mount an immune response to fight infection, causing normal HSCs to differentiate and proliferate in order to replenish the body's immune cells. Proliferative stress induces DNA damage and causes acquisition of somatic mutations in a single HSC. For CH to arise, a mutant HSC must have a selective advantage over the non-mutant stem cell pool. Given that CH is common in elders, development of clones with CH-related mutations may be an inevitable consequence of aging (Figure 1.3).

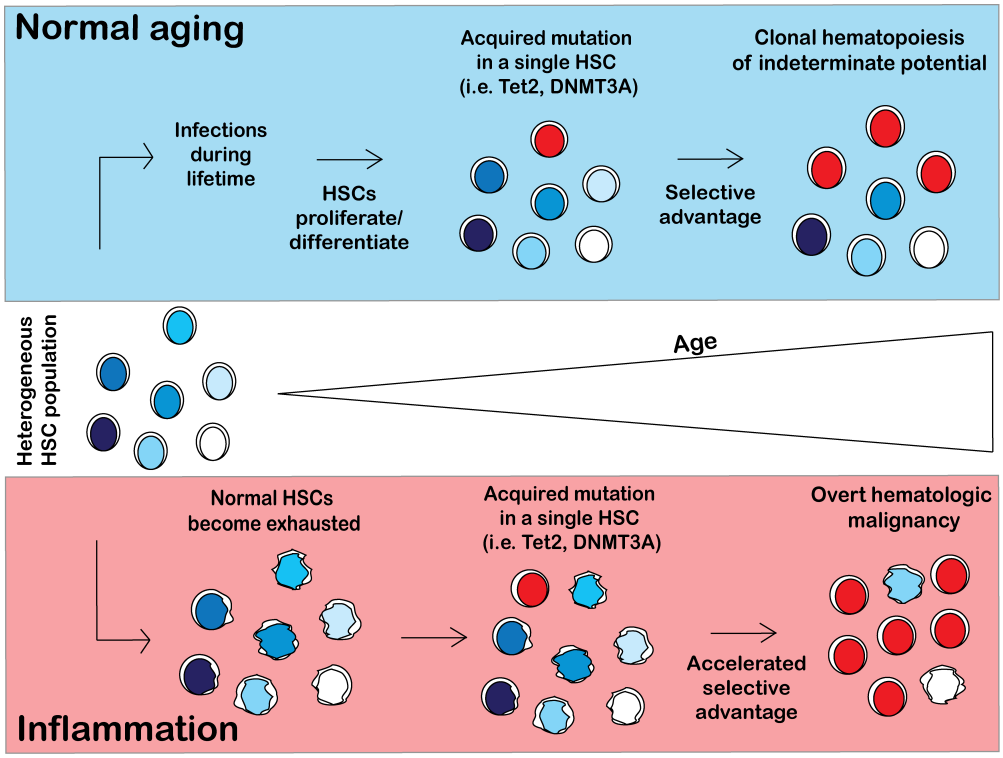

Figure 1.3. Model of clonal hematopoiesis in normal and inflammatory conditions. In a normal person, hematopoietic stem cells (HSCs) will differentiate and self-renew over the course of a lifetime to replenish the blood system. HSCs will acquire somatic mutations as a result of proliferative stress. Certain HSC mutant clones (i.e., *Tet2*, *Dnmt3a*) will acquire a selective advantage over wild type (WT) HSCs, resulting in clonal hematopoiesis. However, in an inflammatory environment, normal HSCs are exhausted and have impaired fitness. HSCs will acquire somatic mutations and will likely have an accelerated selective advantage over the exhausted HSCs, resulting in an earlier onset of CHIP (not illustrated in figure) and possibly in hematologic malignancy.

1.3.2. Aging of Hematopoietic Stem Cells Is Mediated by Inflammation

During aging, human and mouse HSCs increase in frequency but have inferior self-renewal capabilities [69,70]. Aged HSCs also display myeloid skewed differentiation with a decline in lymphopoiesis [71,72]. Compared to younger HSCs, aged HSCs exhibit upregulation of genes associated with the stress response and inflammation and down-regulation of genes involved in the preservation of genomic integrity and chromatin remodeling [73]. In steady state, HSCs are mostly quiescent but in response to hematopoietic challenges such as blood loss, infection, or inflammation, HSCs exit from quiescence to proliferate and increase the output of mature hematopoietic cells [74]. HSC aging can be experimentally hastened by increasing the proliferative history of HSCs through serial transplantation [75,76] or stressing them with multiple injections of myeloablative chemotherapeutic regimens [77].

Chronic inflammation causes HSC aging and leads to HSC exhaustion. Chronic low dose exposure to lipopolysaccharide (LPS), a component of the membrane of gram-negative bacteria, induces HSC to exit quiescence and cycle, increase reactive oxygen species (ROS), and accumulate DNA damage [78–81]. This proliferative stress leads to a reduction in HSC fitness and ultimately HSC exhaustion. Further, mice with sustained *Mycobacterium avium* infection exhibited severe HSC defects as a result of IFNγ-dependent terminal differentiation including pancytopenia and impaired self-renewal and engraftment [79]. HSC that are unable to sense inflammatory signals have superior competitive fitness over wild-type counterparts that retain the ability to respond to inflammatory stimuli [82]. Accelerated aging of HSC from chronic inflammation may promote the emergence of HSC with mutations that endow resistance to inflammation-induced aging (Figure 1.3).

1.3.3. Selective Pressures Will Influence the Outgrowth of Specific Clones

The same principles of natural selection imposed on species can also be applied to populations of cells [83]. Selective pressures from ecological niches fostering the emergence of adaptations in a species are analogous to pressures that occur in stressful hematopoietic microenvironment in which stem cells reside. Selective pressures can be encountered by genetic predispositions (autoimmune diseases), lifestyle (smoking), or therapies from earlier diseases (i.e., cytotoxic agents). The pressures upon an HSC pool dictate which clones will have a selective advantage and emerge, as illustrated in Figure 1.4. The most common mutations associated with CH are in *Tet2* and *Dnmt3a*, which result in defects in HSC differentiation and increased self-renewal [84]. What remains to be seen is whether preserving the fitness of the normal HSC pool may prevent the emergence of neoplastic clones.

Given that clonal hematopoiesis can result from the outgrowth of one or more of 74 known driver genes of leukemia, there is still a paucity of information regarding the mechanism of the outgrowth of the rarer mutant clones, such as $JAK2^{V617F}$. It is likely that less common HSC clones arise in a person in response to a very specific pressure. Similarly, the type of clonal mutation that a person with CH has is likely to inform us of the pressure that was put on their stem cell pool. For instance, JAK2 mutant HSCs are resistant to suppressive growth signaling from chronic TNFα [85]. However, chronic TNFα cannot be the only pressure to select for $JAK2^{V617F}$ clones because TNFα is elevated in most myeloid malignancies (Figure 1.1). A complex environment of elevated cytokines may be required for the outgrowth of $JAK2^{V617F}$, as represented in Figure 1.4. Cell extrinsic influences probably contribute to selective advantage of mutant clones, as it is unlikely that single clone produces enough inflammation to provide selective advantage over normal HSCs.

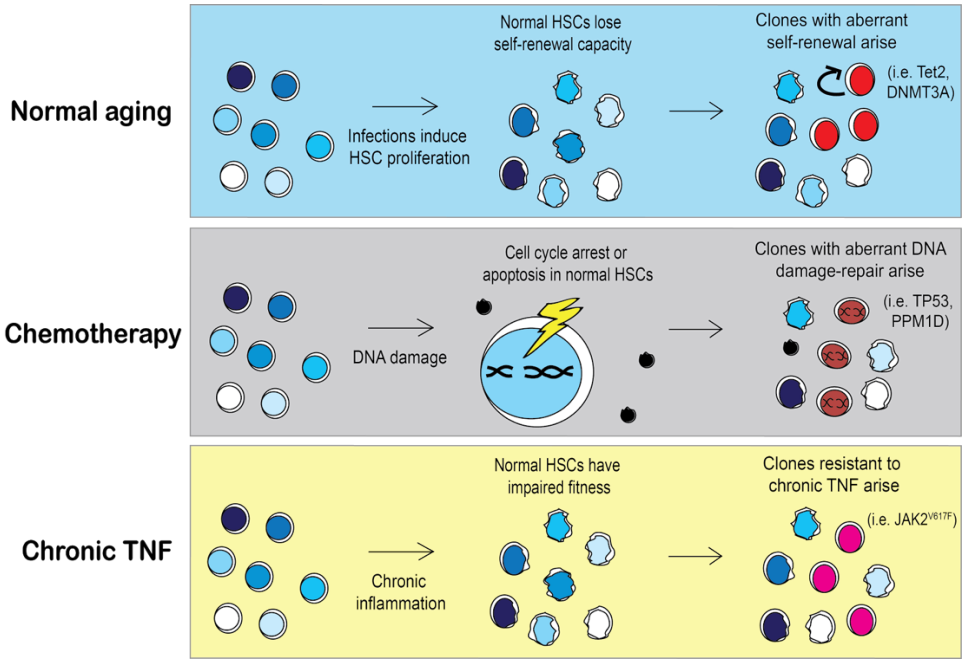

Figure 1.4. Selective pressures may shape clonal expansion of mutant HSCs. During aging HSCs lose their self-renewal capacity as the cells continuously replenish the blood system. As a result, HSCs with mutations that skew towards self-renewal, such as *Tet2* or *DNMT3A*, will have a selective advantage over aged HSCs [84,86]. However, in a person that previously received chemotherapy, HSC clones with different mutations will arise. Chemotherapy induces DNA damage in HSCs and activates DNA damage repair pathways or apoptosis. The incidence of HSCs with mutations in DNA damage repair pathways such as *TP53* or *PPM1D* is significantly higher in people with previous chemotherapy treatment [66]. Specific pressures may allow for the expansion of less common mutations such as $JAK2^{V617F}$. MPN patients with $JAK2^{V617F}$ mutations have elevated TNFα, contributing to the chronic inflammatory environment. Under chronic inflammation, normal HSCs will become exhausted and lose their competitive fitness, allowing for the emergence of mutant HSCs that are resistant to TNFα [85].

1.3.3.1. Chemotherapy and Other DNA Damaging Agents as a Selective Pressure

It is well understood that previous exposure to DNA damaging agents such as chemotherapies or radiation increases the risk of a secondary hematologic malignancy [87]. However, there is growing interest into the exact mechanisms by which chemotherapy or radiotherapy induce secondary hematologic malignancies. One recent study showed that CH is present in approximately 25% of solid-tumor cancer patients and is associated with increased age, prior radiation, and smoking [66]. The incidence of CH was not higher in cancer patients who had previous exposure to chemotherapy over those who were not exposed to chemotherapy; this

suggests that the mutant clones were already present at the time of cancer diagnosis and not from chemotherapy-induced mutations. However, in patients harboring CH mutations and who also had prior exposure to chemotherapy, there was a higher chance that these patients had TP53 or PPM1D mutations rather than DMNT3A or Tet2 clones [66]. This suggests that chemotherapy provides a selective advantage for certain types of HSC clones (i.e., TP53 or PPM1D) and not for other mutant clones (i.e., DNMT3A or Tet2). Another recent study investigated the influence of chemotherapy exposure on the variant allele frequency (VAF) of HSC clones in patients with a variety of CH mutations. In patients that received eight cycles of chemotherapy, the VAF of clones with a DNMT3A mutation remained unchanged after 20 months of treatment. However, the VAF of clones with mutations in the DNA damage-repair protein, RAD21, was increased following chemotherapy [88]. This study confirmed previous data in mouse models showing that DNMT3A mutant HSCs are resistant to anthracycline exposure stemming from an inability to sense and repair DNA torsional stress [89]. Collectively, these findings provide evidence that response of HSCs to DNA damaging agents is mutation dependent. It is possible that the types of mutations that a person with CH harbors may be informative of the selective pressures that were imposed on their HSCs over a person's lifetime.

Hydroxyurea, also known as hydroxycarbamide, is a common cytoreductive agent used in MPN. Hydroxyurea is converted to a free radical nitroxide (NO) in vivo and then transported by diffusion into cells where it inhibits DNA synthesis by inactivating ribonucleotide reductase. Although controversial, long-term use of hydroxyurea may potentially promote progression to leukemia [90]. It is possible that long-term exposure to hydroxyurea compounded with chronic inflammation selects for mutated HSC clones that result in leukemia.

1.3.3.2. Lifestyle Pressures that Influence Inflammation and Hematopoiesis

Lifestyle factors such as obesity and smoking are associated with myeloid malignancy development through the promotion of chronic inflammation. Elevated body mass creates an inflammatory environment and is a risk factor for myeloid malignancy development [91]. Diet-induced obesity modeled by a high fat diet (HFD) changes the composition of adipocyte tissue in the bone marrow and disrupts the ability of mesenchymal progenitors to generate osteoblastic cells [92–94]. Obesity directly harms HSCs, as shown in a HFD model as well as in db/db mice. HSCs from an obese mouse have an exacerbated proliferative response following transplantation, which leads to increased contribution in primary transplants, but negatively impacts competitive ability in secondary transplants [93]. These effects of obesity appear to be driven by upregulation of the gene *Gfi* that is driven by oxidative stress. The effects of obesity on HSC are not reversible with normalization of weight but can be prevented by the antioxidant N-Acetylcysteine [95]. Consistent with these findings is the increased risk of AML [96], MDS [39], and MPN [97] disease development among those with elevated body mass index (BMI) at baseline.

Smoking is a second lifestyle factor that has been associated not only with elevated inflammation [98–100], but with disease development for AML, MDS, CMML and MPN [101–104]. Smoking induces oxidative stress and leads to a chronic inflammatory state, which may act synergistically to promote malignancy. In addition, smokers are more likely to have CHIP [66], suggesting that smoking may play an important role in the very early stages of myeloid malignancy development.

The microbiome is another parameter that affects inflammation and can be manipulated by lifestyle. The human microbiome is defined as the entirety of micro-organisms (including bacteria, viruses, fungi and parasites) living in the human body in a symbiotic interaction where they endorse a protective role most of the time. The microbiome performs digestive and metabolic

functions and is responsible for 40% of the host's energy intake [105]. Besides energy harvest, it plays a role in the development of the host's immune system, as mice lacking gut microbes have altered immune responses [106]. Microbiota dysregulation (or dysbiosis) is seen in a variety of inflammatory diseases including inflammatory bowel disease [107], lupus [108] and rheumatoid arthritis [109]. Viruses and bacteria are also well-recognized triggers of hematologic conditions such as MALT lymphomas induced by *H. pylori* [110]. Thus, targeting or modulating the microbiota may be a novel therapeutic strategy that could complement established treatments for inflammatory conditions.

The microbiome directly modulates innate and adaptive immune responses. For example, germ-free mice infected with *Listeria monocytogenes* have reduced myelopoiesis and differentiation compared to colonized mice [111]. These infected mice die from systemic infection but recolonization of the gut microbiota from colonized mice afforded the mice resistance to infection [111]. Disruption of microbiota-host symbiosis has been implicated as a promoter of colorectal cancer through the induction of inflammatory cytokines by immune cells [112]. Pathobiotic bacteria invade normal colon tissue and induce local inflammation, creating an important link between the gut microbiota and colorectal cancer development [113]. Just as pathobiotic bacteria can induce inflammation and lead to colon cancer, beneficial symbiotic bacteria can reduce inflammation and protect the host from cancer.

Josefsdottir et al. demonstrated an instructive role for the microbiome in hematopoiesis. Depletion of the microbiome in mice with long-term broad-spectrum antibiotics led to impaired hematopoietic progenitor maintenance and granulocyte maturation that was a result of altered T cell homeostasis. The antibiotic induced defects were reversible with fecal microbiota transfer, demonstrating that preservation of the microbiome could be used to prevent antibiotic-associated

bone marrow suppression [114]. New evidence of the interactions between the microbiome, inflammation and blood disorders are growing, opening a new promising field in cancer prevention and therapeutics.

1.3.4. The Bone Marrow Microenvironment and Inflammation

The role of the bone marrow microenvironment on HSC homeostasis has been of considerable interest for several decades. The BM niche involves a complex interaction of multiple cell types including HSCs and stromal cells such as mesenchymal (MSCs) and endothelial cells (ECs) to maintain steady-state hematopoiesis. In the context of inflammation, these interactions are even more critical as MSCs and ECs can detect inflammatory cytokines and engage an "inflammatory loop", harming healthy HSCs and modifying regular hematopoiesis [115]. This inflammatory state was shown to trigger fibrosis and angiogenesis, contributing to the pathogenesis of MPN [116,117]. Other malignancies, such as myelodysplasia and secondary leukemias, can be induced by abnormal osteoprogenitors within the bone marrow niche [118]. Besides its possible role as a driver of the inflammation in hematologic malignancies, the bone marrow microenvironment could also potentiate relapse after chemotherapy and may be a potential therapeutic target for treatment of resistant leukemias [119]. While it is well accepted that the bone marrow microenvironment plays a critical role in myeloid malignancies, it remains to be seen whether the microenvironment promotes the onset of clonal hematopoiesis.

1.4. Future Prospects in the Clinic and on the Bench

There has been a rapid expansion of therapies for hematologic malignancies targeting signaling pathways co-opted by neoplastic cells that allow them to survive and expand. However, much less attention has been paid to the normal HSC pool and the selective pressures that allow

for mutant clones to outcompete their wild type "normal" counterparts. Interventions that preserve or even rejuvenate the fitness of the "normal" HSC pool may neutralize the selective pressure for the neoplastic clones. Chronic inflammation drives HSC aging and leads to a reduction in fitness. Therefore, interventions that reduce inflammation or protect HSC before mutant clones expand may be more impactful than eradicating mutant clones after they have expanded and taken over hematopoiesis. Low-risk interventions with lifestyle adjustments such as diet and exercise may be an ideal approach to reduce inflammation in those individuals with small mutant clones without clinical consequences (i.e., CHIP).

The factors that drive the initial emergence and progression of CHIP to a hematologic malignancy remain undefined, but inflammation likely plays a role in both of these processes. To date, most studies regarding CHIP have consisted of retrospective studies in humans. While mouse models of the most common CHIP associated mutations, including *Tet2*, *DNMT3A*, *PPM1D*, and *JAK2*^{V617F} exist, no models of spontaneously emerging CHIP are present. It is unclear whether CHIP is a common feature of murine hematopoietic aging as it is in humans. If so, it would be of interest to define the external or intrinsic factors that promote or prevent the emergence of CHIP. Mathematical modeling can be used as an approach to help define how specific pressures lead to the selective outgrowth of neoplastic HSC clones. Multiple investigators have modeled the effects of inflammation on MPN mutant versus wild-type HSCs, and this approach could be expanded to test the effect of other selective pressures on both types of HSCs [120,121].

1.5. Conclusions

Our understanding of the role of inflammation in myeloid malignancies continues to expand. In this review, we discussed development, progression and symptom burden of myeloid hematologic malignancies in the context of chronic inflammation. Chronic cytokine stimulation

leads to HSC exhaustion and provides a selective advantage for tolerant mutant clonal stem cells. What remains to be seen, however, is how different types of selective pressures promote the outgrowth of mutated HSCs. Interventions that preserve HSC fitness through reduction of inflammation could potentially prevent initiation and/or progression of myeloid malignancies. Targeting inflammation could be as valuable as targeting the malignant clone, at least for chronic myeloid diseases.

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CHAPTER 2

In Chapter 2, we focus on differential hematopoietic stem cell responses to inflammation in normal versus JAK2 mutant cells. The bacterial component lipopolysaccharide (LPS) is commonly used to induce inflammation and cells directly sense this pathogen-associated molecular pattern via the toll like receptor 4 (TLR4) on stem cells and surrounding stroma. Inflammation is measured by elevated levels of cytokines in the blood or marrow and can be quantified via ELISA-based assays. LPS induces production of TNFα, IL-6 and IFNγ, among other pro-inflammatory cytokines; and physiological effects of LPS include symptoms of weight loss, reduced body temperature and transient leukocytopenia.

Previous reports on the effects of LPS in HSCs have been described. Esplin et al found chronic exposure to LPS injures HSCs by chronically increasing the proliferation of stem cells and causing subsequent fitness impairment (16). This group also found that chronic exposure to LPS increases stem cell mobility to the spleen, and extramedullary hematopoiesis could possibly contribute to the functional stress that HSCs undergo during chronic inflammation (16). Additional studies by Zhang et al and Takizawa et al used transgenic knockout mouse models to show that impaired HSC fitness by chronic LPS is dependent upon the TRIF-ROS-p38MAPK pathway (15,17). Functional impairments correlated with DNA stress response by presence of the DNA damage marker γH2AX (15). Here, we expanded on normal stem cell response to acute LPS using a time course of 2-48 hours and found that HSCs enter cycling earlier than multipotent progenitors. In this chapter, we compare stem cell response as measured by proliferation, oxidative stress and DNA damage via γH2AX in wildtype versus JAK2 mutant stem cells following LPS as well as the functional impact as measured by competitive transplantation.

Article

Differential response of JAK2 mutant hematopoietic stem and progenitor cells to Toll-like receptor 4 activation

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Subject Ontology: stem cells, inflammation, myeloproliferative neoplasm

Abstract

Chronic inflammation is a characteristic feature of myeloproliferative neoplasm (MPN), yet how $JAK2^{V617F}$ hematopoietic stem cells (HSC) respond differently than normal HSC to inflammatory insult remains unclear. First, in wild-type (WT) mice we established with fine resolution the time course of events that occur following exposure to lipopolysaccharide (LPS), a common experimental model of inflammation LPS stimulation, with plasma TNFα peaking by two hours post-LPS stimulation, then reactive oxygen species (ROS), proliferation, and DNA damage peaking at 16 hours. HSC began proliferating at 8 hours post-LPS stimulation, prior to induction of ROS, however in progenitors (MPP2/3) proliferation did not occur prior to ROS, suggesting that LPS may induce ROS independent proliferation in HSC. We next compared the response of *Jak2*^{V617F} knock-in versus WT hematopoietic stem and progenitors to LPS. In the absence of LPS stimulation *Jak2*^{V617F} HSC were more proliferative than WT, but with LPS stimulation *Jak2*^{V617F} and WT HSC were equally proliferative.

Introduction

Hematopoietic stem cells (HSC) respond to the need for increased mature hematopoietic cells during infection by proliferating in response to inflammatory cues. However, chronic inflammation leads to proliferation induced exhaustion of HSC. A common theme among myeloid malignancy associated mutations is that their selective advantage is augmented in the context of chronic inflammation(1-3) suggesting that HSCs with myeloid malignancy associated mutation respond differently than wild-type HSC to inflammatory cues. Defining the impact of inflammation on HSC fitness may reveal insights into how myeloid malignancy associated mutant HSC gain a selective advantage in the context of inflammation, allowing them to expand resulting in hematologic malignancy.

JAK2^{V617F} is the most common MPN driver mutation and elicits constitutive JAK/STAT signaling within the HSC clone and its progeny. We have previously shown that *Jak2*^{V617F} hematopoietic progenitors are resistant to growth inhibition by the inflammatory cytokine TNFα, though it is unclear whether this phenomenon persists in the HSC population(2).

Lipopolysaccharide (LPS) is commonly used as an inflammation model and directly induces Toll-like receptor (TLR) 4 signaling in HSCs. TLR activation leads to increased reactive oxygen species (ROS), activation of p38MAPK and NF-κB signaling pathways, ultimately causing proliferation-induced stem cell exhaustion(4-6). Following an LPS challenge HSCs exhibit an increased DNA damage response as measured by phosphorylation of ser139 on histone 2A, forming γH2AX foci, this is dependent upon activation of the TRIF-ROS-p38 pathway(4). Here, we investigated how *Jak2*^{V617F} HSCs respond to LPS with respect to proliferation, oxidative stress, and DNA damage. We found that *Jak2*^{V617F} stem and progenitor cells are more proliferative than wild-type cells at baseline but in response to stimulation with LPS are less responsive than wild-type. We also assessed the functional impact of LPS on HSC in competitive transplantation assays

and found that the antioxidant N-Acetylcysteine protects HSC from the reduction in fitness usually seen after chronic LPS exposure.

Results

Identification of key time points in the physiologic response following acute inflammatory stimulus

To finely resolve the temporal response of wild type (WT) HSCs to acute inflammation we analyzed mice at multiple time points from 2 to 48 hours following a single 10μg intraperitoneal injection of LPS. A reduction in white blood count was seen by 2 hours post-LPS (Supplemental Fig 2.1A), and a decrease in body weight was evident by 4 hours post-LPS (Supplemental Fig 2.1E). Plasma TNFα levels peaked at 2 hours post-LPS stimulation and returned to near baseline by 24 hours post-LPS (Fig. 2.1B). Total BM cellularity was reduced over time and the frequencies of LKS-SLAM ($Lin^{-}ckit^{+}Sca1^{+}CD150^{+}CD48^{-}$) and MPP2/3 ($Lin^{-}ckit^{+}Sca1^{+}CD48^{+}$) cells increased with LPS (Fig. 2.1C-E). However, the absolute numbers of LKS-SLAM and MPP2/3 remained stable (Supplemental Fig. 2.1I-K). These data suggest that the increase in frequency in hematopoietic stem and progenitors (HSPC) following LPS stimulation is from a decrease in mature cells in the bone marrow rather than an increase in the absolute number of stem and progenitor cells.

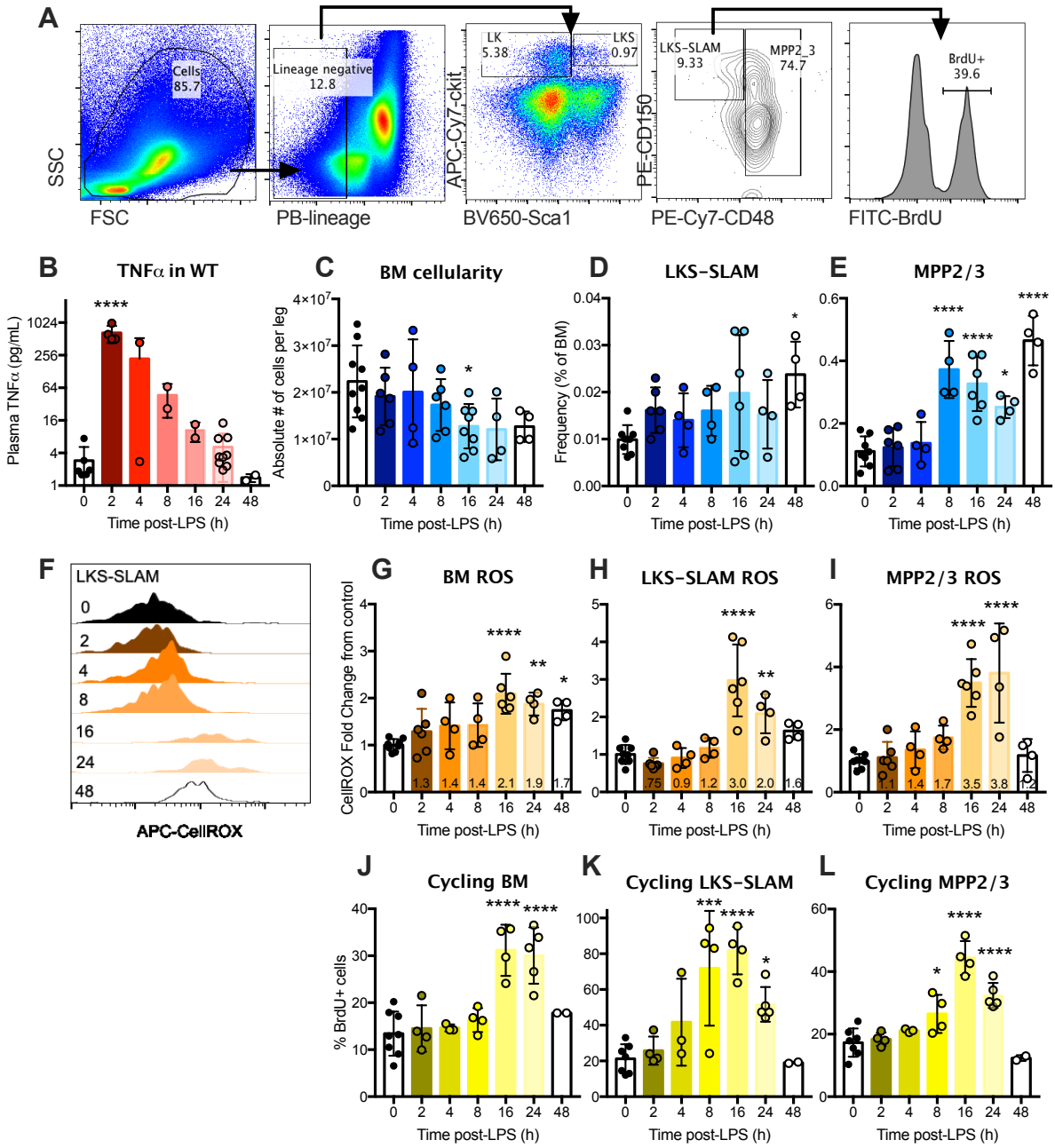

Figure 2.1. Time course of normal hematopoietic stem cell and progenitor frequency, oxidative stress and cycling following acute inflammation. A. Representative gating for bone marrow cells (BM), HSCs (LKS-SLAM) and multipotent progenitors (MPP2/3). B. TNFα ELISA from mouse plasma collected at various time points post-LPS treatment. C. Absolute number of BM per leg. D-E. Frequency of (D) LKS-SLAM and (E) MPP2/3 of total BM cells. F. Representative CellROX histograms gated on LKS-SLAM. G-I. Fold change of CellROX mean fluorescence intensity normalized to untreated control in (G) BM, (H), LKS-SLAM, and (I) MPP2/3 cells. J-L. Percentage of cells with BrdU incorporation in (J) BM, (K) LKS-SLAM and (L) MPP2/3 cells. Error bars represent SD, n=4-8/group; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; one-way ANOVA.

We measured ROS using the cell permeant dye CellROX and observed a significant increase in CellROX fluorescence intensity in all cells of interest, with ROS peaking at 16-24 hours post-LPS (Fig. 2.1F-I). LPS-induced oxidative stress promotes exit from quiescence leading to proliferation-induced exhaustion in wild-type HSCs(4). We used Bromodeoxyuridine (BrdU) incorporation to measure proliferative history and found the peak of BrdU incorporation to be at 16 hours post-LPS in all cell populations tested, however LKS-SLAM exhibited increased BrdU incorporation as early as 8 hours post-LPS (Fig. 2.1J-L). These data corroborate the notion that ROS induces proliferation of HSPC, however in LKS-SLAM proliferation may occur even prior to ROS induction. All together, these data confirm that LPS is a potent inducer of inflammation in vivo and increases HSPC proliferation and oxidative stress.

Inflammation, oxidative stress, and proliferation in wild type versus Jak2V617F-mutant HSPCs

Next, we asked whether *Jak2*^{V617F} HSPC respond differently than WT to acute LPS exposure. At 16-24 hours post LPS injection bone marrow cells from *Jak2*^{V617F} knock-in and WT mice were harvested to quantify HSPC frequency and absolute number, to measure their proliferative history using BrdU incorporation, and to measure ROS. We also measured plasma TNFα of these mice. At baseline the TNFα plasma concentration was slightly elevated in *Jak2*^{V617F} mice as compared to WT mice. However, in *Jak2*^{V617F} mice LPS stimulation failed to significantly increase plasma concentration of TNFα, this could be reflective of a chronic inflammatory state being less responsive to additional stimuli (Fig. 2.2A). LPS exposure resulted in an increased frequency of LKS-SLAM and MPP2/3 similarly in *Jak2*^{V617F} and WT mice (Fig. 2.2B-C). LPS increased ROS in all hematopoietic cell populations tested from WT and *Jak2*^{V617F} knock-in BM (Fig. 2.2D-F). At baseline, *Jak2*^{V617F} knock-in MPP2/3 had significantly higher ROS (5-fold)

compared to WT (normalized to 1, P<0.0001, one-way ANOVA; Fig. 2.2F). We used BrdU incorporation to compare the proliferative history of WT versus *Jak2*^{V617F} HPSCs in response to LPS. LPS exposure led to a significant increase in BrdU^{+} LKS-SLAM cells in WT but not *Jak2*^{V617F} knock-in mice (Fig. 2.2G-I), as *Jak2*^{V617F} mice had an increased percentage of BrdU^{+} LKS-SLAM even without LPS. This demonstrates that *Jak2*^{V617F} LKS-SLAM cells are more proliferative than WT without exogenous inflammatory stimuli, however they are less apt to increase cycling with external inflammatory cues.

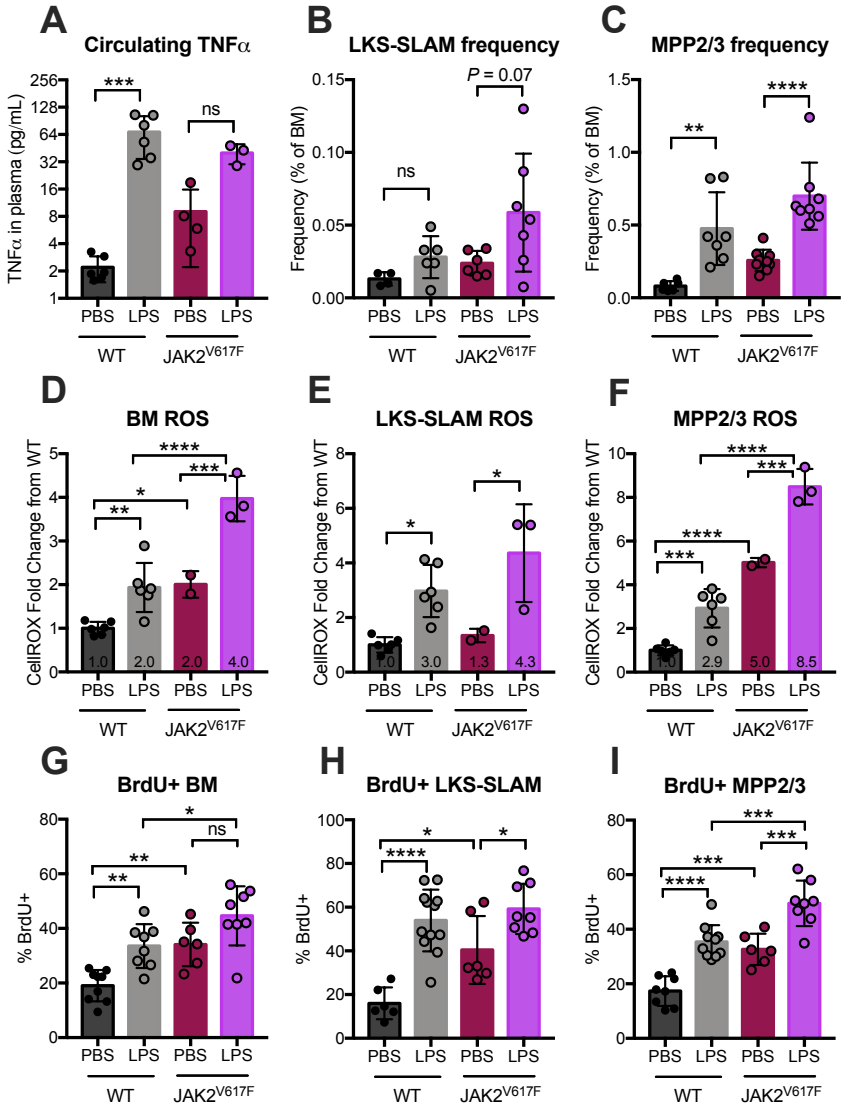

Figure 2.2. Normal versus JAK2^{V617F} hematopoietic stem cell and progenitor frequency, oxidative stress and cycling following acute inflammation. A. TNFα ELISA from mouse plasma. B-C. Frequency of (B) LKS-SLAM and (C) MPP2/3 of total BM cells. D-F. Fold change of CellROX mean fluorescence intensity normalized to wildtype (WT) PBS control in (D) BM, (E), LKS-SLAM, and (F) MPP2/3 cells. G-I. Percentage of cells with BrdU incorporation in (G) BM, (H) LKS-SLAM and (I) MPP2/3 cells. Error bars represent SD, n=2-8/group; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; one-way ANOVA.

Distinct γH2AX incorporation in HSCs versus multipotent progenitors following LPS-induced inflammation

Inflammation induces HSCs to exit quiescence, activating the DNA damage response(4, 7). We established the time course of γH2AX accumulation in WT HSC following LPS stimulation using flow cytometry. The mean fluorescence intensity (MFI) of γH2AX peaked in LKS-SLAM cells at 8 hours post-LPS and peaked in MPP2/3 cells at 16 hours post-LPS (Fig. 2.3A-C). The trend of γH2AX increase correlated with BrdU incorporation in all cell types (Fig. 2.1I-K and Fig. 3A-C) consistent with the notion that proliferation increases γH2AX. To directly observe γH2AX foci localization and compare LPS-induced DNA damage in WT versus *Jak2^{V617F}* cells, we sorted LKS-SLAM and MPP2/3 cells from control or LPS-treated mice and performed γH2AX immunofluorescence on fixed cells (Fig. 2.3D, E). Indeed, LPS-treated LKS-SLAM cells exhibited increased γH2AX staining compared to controls, however distinct foci were not observed (Fig. 2.3D). This suggests that the rapid increase in γH2AX measured by flow cytometry represents cell cycling rather than damaged DNA and may account for the correlation of BrdU and γH2AX over time. On the other hand, MPP2/3 cells exhibited distinct γH2AX foci following LPS treatment which may represent DNA damage (Fig. 2.3E). While *Jak2^{V617F}* knock-in cells accumulated DNA damage even in unstimulated controls, γH2AX foci were increased even further following LPS exposure (representative images in Fig. 2.3D-E, quantification in Supplemental Figure 2.3D-E).

These observations suggest that the DNA-damage marker γH2AX is rapid and dynamic in cycling HSCs and may be differentially regulated in stem versus progenitor cells.

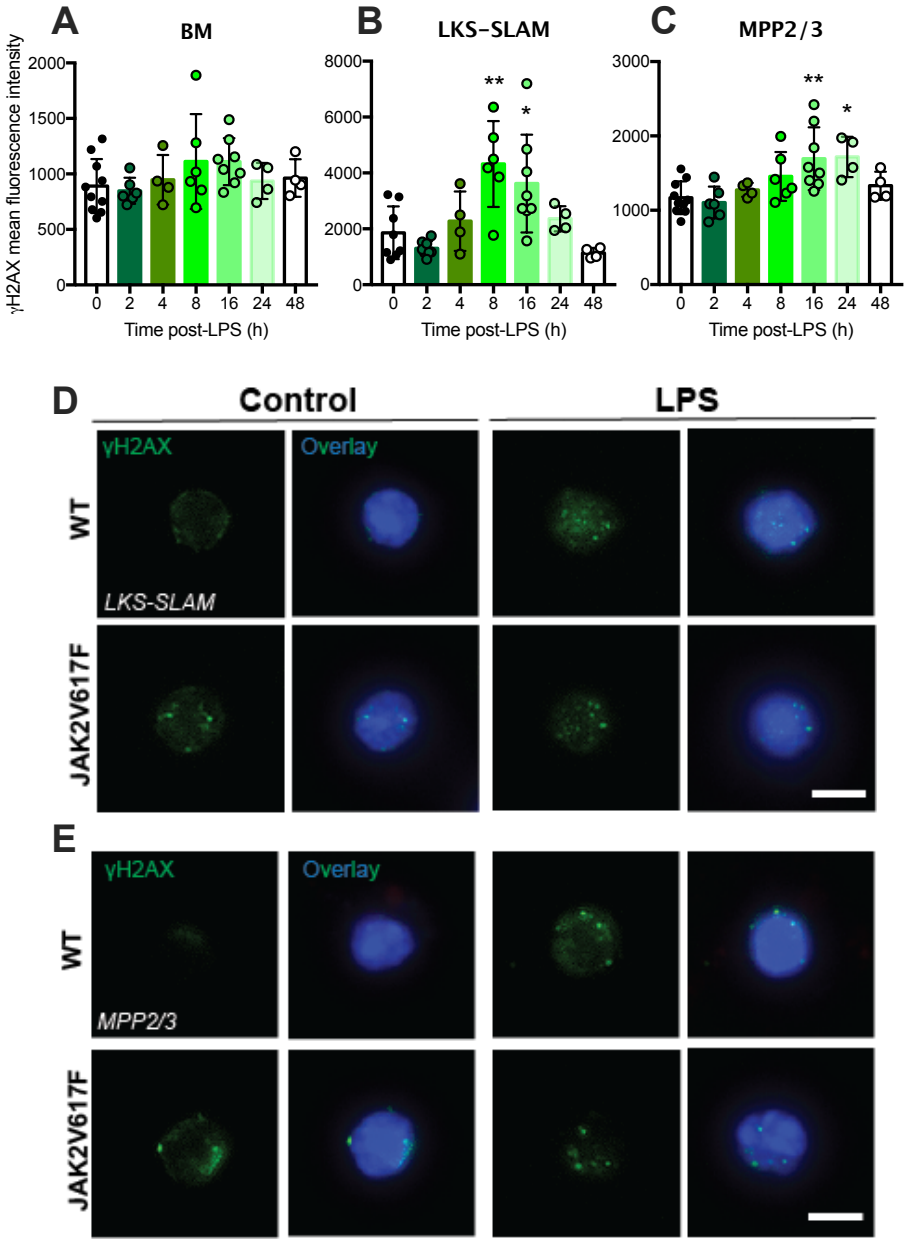

Figure 2.3. Unique γH2AX foci in normal and mutant HSCs versus MPPs following LPS-induced inflammation. A-C. Time course of γH2AX mean fluorescence intensity in (A) BM, (B), LKS-SLAM and (C) MPP2/3 cells via flow cytometry. Error bars represent SD, n=4-8/group; **P*<0.05, ***P*<0.01; one-way ANOVA. D-E. Representative immunofluorescence images of sorted (D) LKS-SLAM and (E) MPP2/3 cells from WT and JAK2^{V617F} knock-in mice +/– LPS treatment. DAPI (blue) and γH2AX (green), 60× magnification; scale bar represents 5μm.

Response of Jak2^{V617F} and WT HSPC residing in same bone marrow to inflammatory stimuli

In MPN patients *JAK2^{V617F}* and WT HSPC co-exist in the bone marrow, the presence of *JAK2^{V617F}* cells may change how their WT neighbors respond to inflammatory stimuli. To investigate the effect of inflammation on WT versus *Jak2^{V617F}* hematopoietic cells residing in the same environment, we developed chimeric mice by transplanting equal numbers of whole BM cells from WT (CD45.1) and *Jak2^{V617F}* (CD45.1/2) mice into lethally irradiated WT (CD45.2) recipients (referred to as WT:VF hereafter). We also transplanted whole BM cells from WT (CD45.1) and WT (CD45.1/2) into lethally irradiated WT (CD45.2) recipients (referred to as WT:WT hereafter). At 16 weeks post-transplant, mice were injected with LPS then HSCs were harvested and stained with CD45.1 and CD45.2 antibodies to assess WT and *Jak2^{V617F}* (VF) HSPCs separately (Fig. 2.4A).

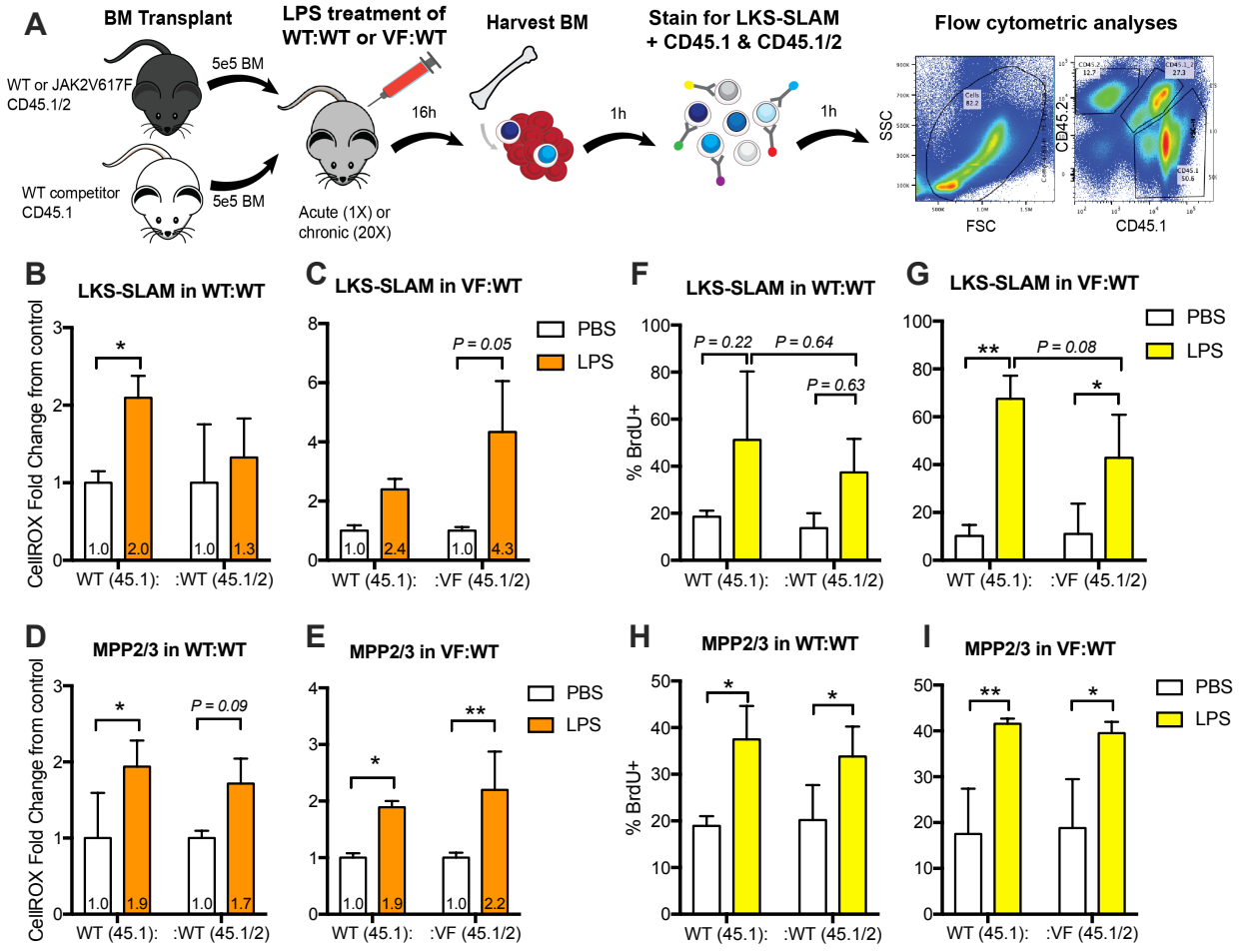

Figure 2.4. Cell-intrinsic differences between WT and JAK2^{V617F} response to acute inflammation. A. Schematic of transplant. B-E. CellROX Mean Fluorescence Intensity (MFI) of LKS-SLAM or MPP2/3 cells from (B, D) mice transplanted only with WT cells (WT:WT) and (C, E) mice transplanted with JAK2^{V617F} and WT cells (VF:WT). F-I. Percentage of BrdU+ LKS-SLAM or MPP2/3 cells from (F, H) WT:WT transplanted mice and (G, I) VF:WT transplanted mice. Error bars represent SD, n=2-4/group; **P*<0.05, ***P*<0.01; 2way ANOVA.

Mice transplanted with only WT cells, referred to as WT:WT, had comparable concentrations of plasma TNFα as mice transplanted with a mixture of VF and WT cells (VF:WT, Supplemental Fig. 2.2). HSCs from WT:WT mice demonstrated a dampened response to acute LPS stimulation (Fig 2.4F) as compared to non-transplanted WT mice (Fig 2.1K). The increased proliferation of VF HSCs without LPS stimulation was not observed in transplanted mice (Fig 2.4G). This demonstrates that transplantation alters the response of HSC to acute inflammatory stressors, making it difficult to directly compare transplanted versus non-transplanted mice.

Therefore, we focused our comparisons on WT versus *Jak2*^{V617F} residing in the same environment (CD45.1 vs CD45.1/2 in WT:VF mice). *Jak2*^{V617F} and WT HSPC co-existing in the same transplanted mouse behaved similarly with respect to proliferative history (BrdU uptake) (Fig 2.4G,I). *Jak2*^{V617F} LKS-SLAM had a more intense increase in ROS in response to LPS (4.3 fold) as compared to their WT neighbors (2.4 fold), however ROS was similarly induced in the MPP2/3 compartment (2.2 fold in *Jak2*^{V617F} vs 1.9 fold in WT). The impact of transplantation on the response of HSC to inflammatory cues may blur the ability to detect a significant bystander effect of *Jak2*^{V617F} cells on their WT HSC neighbors.

Differential effect of N-acetylcysteine on wildtype and Jak2^{V617F} *HSCs*

Next, we investigated how the ROS scavenger N-acetylcysteine (NAC) impacts HSC response to inflammation (Fig. 2.5). Oral administration of NAC (2gm/L in drinking water) reduces LPS-induced inflammation *in vivo* as measured by TNFα ELISA (Fig. 2.5A), consistent with NAC's known anti-inflammatory properties(8, 9). We observed similar ROS in HSC from mice exposed to LPS on drinking water with NAC versus plain drinking water (Fig 2.5B), and also found no difference in the percentage of BrdU^{+} HSC nor ɣH2AX intensity between mice on NAC versus plain water, suggesting that higher doses may be necessary to significantly impact ROS at the HSC level.

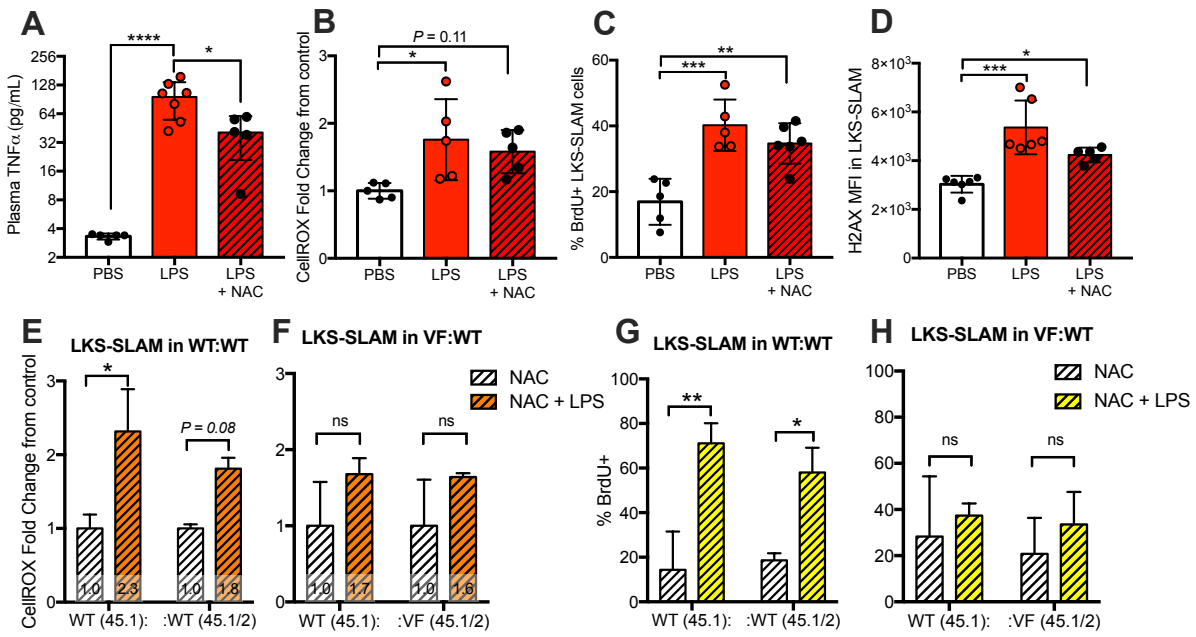

Figure 2.5. Effect of the antioxidant N-acetylcysteine (NAC) on naive versus transplanted HSC responses to inflammation. A. TNFα ELISA from WT mouse plasma collected 16h post-LPS +/– NAC treatment. B. Fold change of CellROX MFI normalized to untreated control in LKS-SLAM. C. Percentage of cells with BrdU incorporation in LKS-SLAM. D. Mean fluorescence intensity of γH2AX in LKS-SLAM. Error bars represent SD, n=5-7/group; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; one-way ANOVA. E-F. CellROX MFI of LKS-SLAM from mice transplanted with (E) WT:WT or (F) VF:WT. G-H. Percentage of cycling LKS-SLAM cells from mice transplanted with (G) WT:WT or (H) VF:WT. Error bars represent SD, n=2-4/group; **P*<0.05, ***P*<0.01, 2way ANOVA.

To better understand the role of oxidative stress in a bone marrow environment where *Jak2*^{V617F} and WT cells coexist, we generated chimeric VF:WT mice by transplanting equal ratios of WT and *Jak2*^{V617F} BM into recipients then began NAC treatment 4 weeks post-transplant (2g/L in drinking water). After 4 weeks of NAC treatment mice were exposed to a single dose of LPS (or PBS) 16 hours prior to sacrifice. We observed that NAC had a stronger effect on dampening LPS-induced ROS in HSCs from VF:WT than from WT:WT mice (Fig. 2.5E-F), perhaps suggesting that lower doses of NAC may be sufficient to reduce ROS in environments containing *Jak2*^{V617F} mutant cells. LPS exposure did not induce a significant induction of ROS in VF:WT mice on NAC water (Fig 2.5F), and these mice did not have a significant increase in BrdU^{+} HSC in response to LPS (Fig 2.5H). These data are consistent with the notion that ROS induces HSC

exit from quiescence and suggest that inhibiting ROS and thus HSC cycling in the context of chronic inflammation may prevent proliferation induced exhaustion of HSC.

N-acetylcysteine spares HSCs from chronic-LPS induced exhaustion

To investigate how chronic inflammation with and without antioxidant supplementation impacts HSC function, we exposed mice drinking either plain water or NAC water (2g/L) to chronic LPS (5μg daily x 30 days) and then performed competitive transplants with whole bone marrow at a 1:1 ratio and tracked peripheral blood chimerism over time (Fig. 2.6A). As expected, chronic exposure to LPS impairs wild type HSC competitive fitness (Fig. 2.6B-C). However, when mice were exposed to chronic LPS on NAC water HSC fitness was rescued and comparable to untreated controls (Fig. 2.6D-F). We followed these mice for 6 months, at 6 months post-transplant there was a significant reduction in engraftment of cells exposed to chronic LPS compared to untreated controls (25% versus 55% engraftment, $P<0.001$ t-test; Fig. 2.6G). Competitors exposed to chronic LPS in the presence of NAC exhibited similar competitive fitness as untreated controls (48% versus 55% engraftment, Fig. 2.6G) and had improved fitness compared to competitors exposed to chronic LPS alone (70% versus 47% engraftment, $P<0.05$ t-test; Fig. 2.6H).

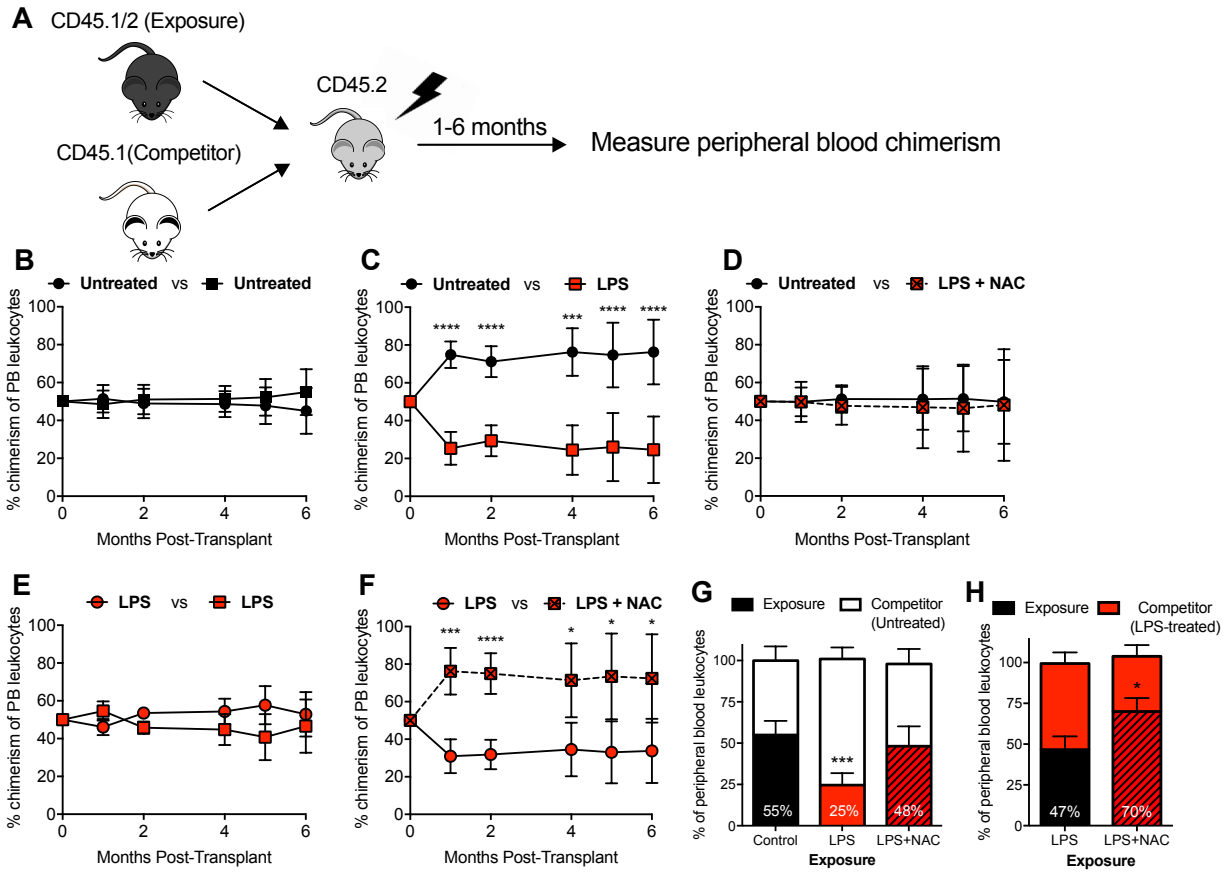

Figure 2.6. NAC spares HSCs from chronic LPS-induced impaired fitness. A. Schematic of competitive transplant where BM from donor mice were transplanted at equal ratios into lethally irradiated recipients. B-F. Peripheral blood chimerism of donor-derived cells where circles represent cells from competitor group (CD45.1) and squares represent cells from exposure group (CD45.1/2). G-H. Peripheral blood chimerism at 24-weeks post-transplant where CD45.1 competitor was (G) untreated or (H) LPS treated. n=2-4/group, error bars represent SD, **P*<0.05, ***P*<0.01; unpaired t-test.

Discussion

Clonal hematopoiesis (CH), which can progress to hematologic malignancy, is a common consequence of aging. HSC with CH associated mutations may gain a selective advantage specifically when the fitness of their wild-type counterparts has decreased, such as from chronic inflammation associated with aging. Specific mutations may provide resistance to specific types of HSC stressors; thus, the stressor may dictate which mutant clones gain a selective advantage

and emerge as CH. *JAK2*^{V617F} clonal hematopoiesis is less common among the general aging population but clusters within families(10). This suggests that a specific stressor present among members of MPN families selects for *JAK2*^{V617F} mutant cells.

Here, we compared the response of *Jak2*^{V617F} mutant versus WT to a common experimental model of inflammatory stress, LPS. HSC from *Jak2*^{V617F} knock-in mice were more proliferative than WT HSC at baseline (Fig 2.2H), however after LPS stimulation proliferation in *Jak2*^{V617F} HSC and WT HSC was equivalent (Fig 2.2H). The increased proliferation at baseline may lead to accelerated exhaustion of HSC and explain why in some experimental mouse models *Jak2*^{V617F} HSC have reduced function in competitive repopulation assays(11). However, chronic inflammation may negatively impact the fitness of WT more than *Jak2*^{V617F} HSC, thus "leveling the playing field" for *Jak2*^{V617F} cells under chronic inflammatory stress. However, it is likely that a very specific type of inflammatory stressor is required to reveal a significant selective advantage of *Jak2*^{V617F} mutant cells. For example, we have observed defective IL-10R signaling among MPN patients(12) which prolongs TNFα production monocytes following LPS stimulation. Chronic inflammation in the context of IL-10R blockade may create an environment that is highly selective for *JAK2*^{V617F} mutant HSC.

We utilized lethally irradiated mice transplanted with a mixture of *Jak2*^{V617F} and WT donor bone marrow to simulate an environment in which *Jak2*^{V617F} and WT cells are co-existing. Not unexpectedly, transplantation impacted the response of HSC even in mice transplanted with only WT bone marrow. Stressed or aged HSC don't respond as well to LPS as young HSC(6). A direct comparison of WT cells residing in an environment containing *Jak2*^{V617F} neighbors versus WT cells residing in an environment with only WT cells did not reveal significant differences in the response to LPS, however the impact of transplantation may mask subtle effects of *Jak2*^{V617F} on

bystander WT HSC (Fig.2.7). This highlights the need for alternative approaches to create murine models that recapitulate the coexistence of mutant and WT cells without the need for myeloablative conditioning regimens, such as low dose irradiation or using c-kit drug conjugate antibodies(13).

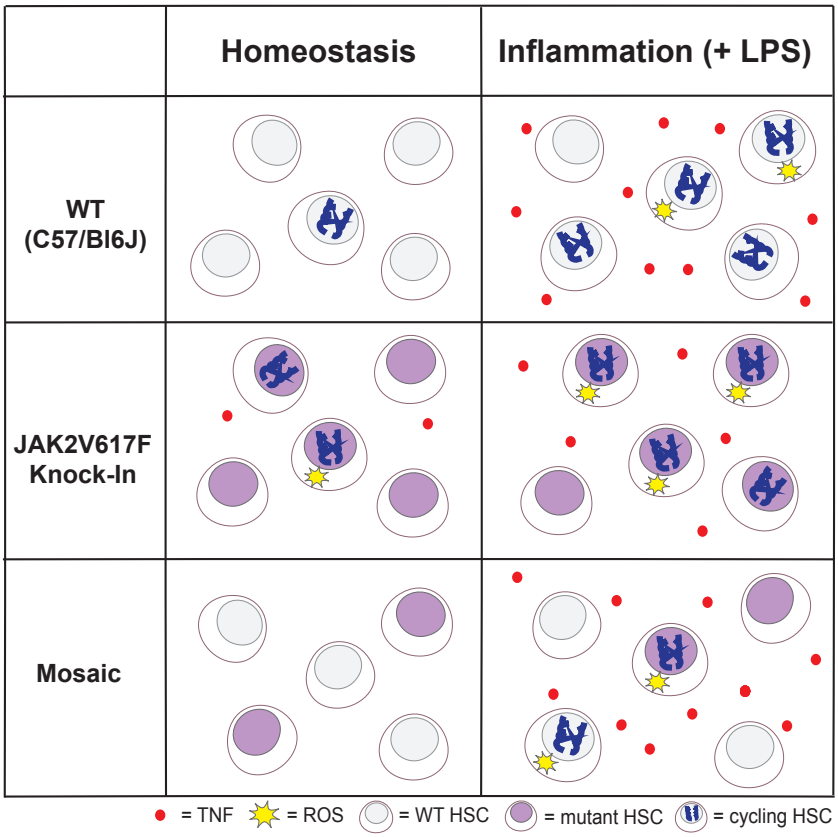

Figure 2.7: Differential effects of acute inflammation on wildtype versus JAK2^{V617F} hematopoietic stem cell response. Most wildtype HSCs are in quiescence under homeostatic conditions. LPS increases inflammation via upregulated TNFα, causing most HSCs to enter S phase and increase oxidative stress. The JAK2^{V617F} mutation causes significant increases in HSC cycling, as well as minor increases in ROS and TNFα under homeostatic conditions. Thus, there is not a significant increase in stem cell cycling upon acute inflammation. Mosaic mice contain transplanted WT and mutant HSCs, which exhibit a diminished response to LPS-induced inflammation. LPS = lipopolysaccharide, WT = wildtype mouse strain C57Bl6/J, TNF = tumor necrosis factor, ROS = reactive oxygen species, HSC = hematopoietic stem cells.

We found that in general ROS peaked at the same time point as proliferation, consistent with the notion that ROS induces HSC to exit quiescence(4). However, in HSC we observed proliferation even before elevation of ROS. HSCs can cycle independently of increased ROS

possibly through other mechanisms such as intracellular calcium levels(14, 15). Consistent with ROS inducing exit from quiescence, when the antioxidant NAC was given at a dose sufficient to reduce ROS a reduction in proliferation is also seen (Fig 2.5). Moreover, treatment with NAC protects HSC from inflammation induced exhaustion (Fig 2.6). Preserving the fitness of WT HSC during aging with antioxidants such as NAC may be beneficial for the prevention of CH and subsequent hematologic malignancy.

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Authorship Contribution: B.M.H. performed experiments, analyzed the data, made the figures and wrote the manuscript; G.R. performed and designed experiments and wrote the manuscript; S.H., J.C., T.T., H.N. and H.Y.L. performed experiments; A.G.F. planned, designed and coordinated the research and wrote the manuscript. All authors read the manuscript and approved its content.

Conflicts of Interest Disclosure: The authors report no conflicts of interest to disclose.

Materials and Methods

Mice

All animal procedures were performed under approval of the Institutional Animal Care and Use Committee at the University of California, Irvine. Equal numbers of male and female mice were used for all experiments. *Vav-Cre:Jak2*^{V617F} murine model were a gift from Ann Mullally(16). The TET2 knockout(17) and DNMT3A^{R878H} mutant mice(18) have been previously

described and were obtained from The Jackson Laboratory ($TET2^{-/-}$, JAX stock #023359 and $Dnmt3a^{fl\text{-}R878H}$, JAX stock #032289) Complete blood counts were measured using the ABCVet Hemalyzer (scil, Viernheim ,Germany) using blood diluted 1:1 with 100mM EDTA. For NAC treatment, mice were given drinking water with 2gm/L NAC (Bulk Supplements, Henderson, NV) beginning at 4 weeks of age or post-transplant and this was replenished weekly.

Inflammation Model

Mice were injected at 0.5-1.5mg/kg intraperitoneally (IP) with lipopolysaccharide from Escherichia coli O55:B5 (cat#L2880, Sigma) in the presence or absence of BrdU (1mg IP). Following the desired time, mice were sacrificed, plasma was harvested for TNFα measurement, bone marrow was flushed and quantified, then stained with stem cell markers for analysis via flow cytometry. TNFα enzyme-linked immunosorbent assays (ELISAs) were performed according to the vendor's protocol (Ready-Set-GO TNFα ELISA, Thermo Fisher).

Flow Cytometry

All antibodies were purchased from BioLegend (San Diego, CA). Whole bone marrow was harvested from both femurs and tibias, RBCs were lysed with Ammonium-Chloride-Potassium (ACK) buffer, washed, then cells were stained for 30 minutes on ice. Pacific blue conjugated antibodies against Ter119, Mac1, Gr1, B220, CD3, CD4 and CD8 were used to detect mature cells (Lineage cocktail). HSC populations were detected by staining with mouse specific antibodies against Lineage markers, c-Kit, Sca-1, CD48 and CD150 (LKS-SLAM or MPP2/3). For BrdU, vendor's protocol was followed (BD Biosciences, San Jose, CA). For ɣH2AX analysis, BrdU fix/perm reagents (BD Biosciences, San Jose, CA) were used for intracellular staining with FITC-H2AX (Biolegend, San Diego, CA). Flow cytometry was performed on the Novocyte (Agilent,

Santa Clara, CA) at the UCI Immunology Core facility. Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Immunofluorescence

Immunofluorescence for ɣH2AX was performed as previously described(7, 19). Briefly, BM cells were harvested and stained with the HSC markers previously listed. LKS-SLAM or MPP2/3 cells were sorted into Eppendorf tubes containing RPMI using a FACSAria Fusion (BD, San Jose, CA) then were cytospun onto poly-lysine coated glass slides and fixed with 4% PFA for 10 min at room temperature. Cells were permeabilized with 0.15% Triton X-100 for 2 min at room temperature then were blocked in 1% BSA in PBS overnight at 4°C. Slides were incubated for 2h at 37°C in 1% BSA with anti-phospho-H2AX (Ser 139) (05-636; Millipore, Burlington, MA) then were washed in PBS and incubated for 1h at 37 °C in 1% BSA with A488-conjugated donkey anti-mouse (A-21202, Thermo Fisher). Slides were washed three times in PBS then incubated with DAPI for 5 min before mounting with Permount. Images were taken with a Keyence microscope using 60✕ objective with oil immersion.

Competitive transplants

Bone marrow was harvested from donor mice (CD45.2) exposed to either PBS or LPS. B6.SJL-Ptprca Pepcb/Boy (BoyJ, CD45.1) mice were purchased from The Jackson Laboratory. Recipient mice (CD45.1/CD45.2) were lethally irradiated with 8 Gy the day before transplantation. To develop chimeric mice, donor whole bone marrow cells from WT C57Bl/6J or $JAK2^{V617F}$ knock-in mice (CD45.2) were mixed in a ratio of 1:1 ($1x10^6$ each) with competitor cells (CD45.1) and injected intravenously into irradiated recipients. Mice were rested for 16 weeks before beginning LPS exposures. Flow cytometry for chimerism of mature cells in peripheral blood was

performed using CD45.1 and CD45.2 antibodies. Data was acquired on the BD Accuri C6 (BD Biosciences, San Jose CA).

Statistical Analysis

All statistical analyses were performed using Graphpad Prism version 7.0 (Graphpad, La Jolla, CA, USA). Data are shown as mean and error bars indicate the standard deviation (SD). P-value was calculated using a one-way ANOVA (Sidak's test; Fig. 1-3, 5A-D), 2way ANOVA (Sidak's test; Fig. 4 & 5E-H) or an unpaired t-test (Holm-Sidak; Fig. 6) comparing the means of two groups. Significant differences were indicated as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. All experiments were performed at least three independent times unless otherwise stated.

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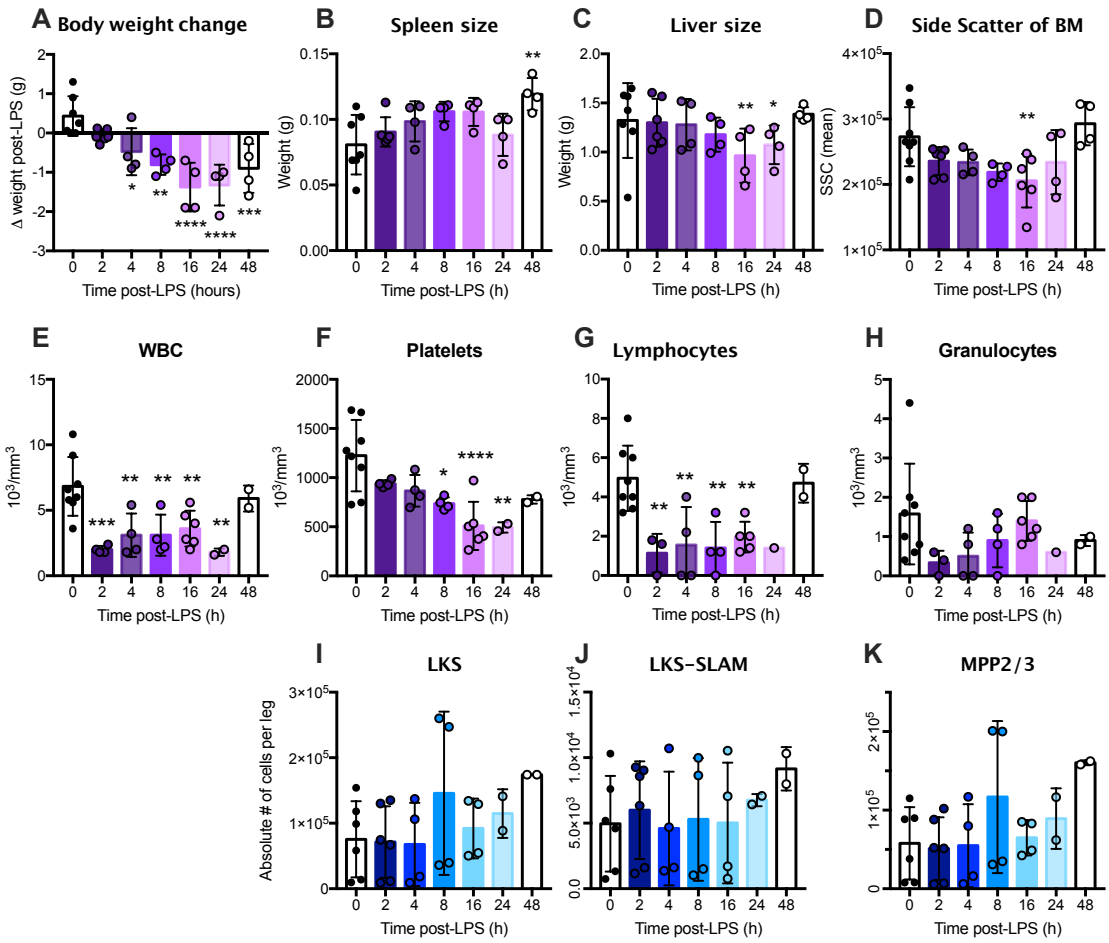
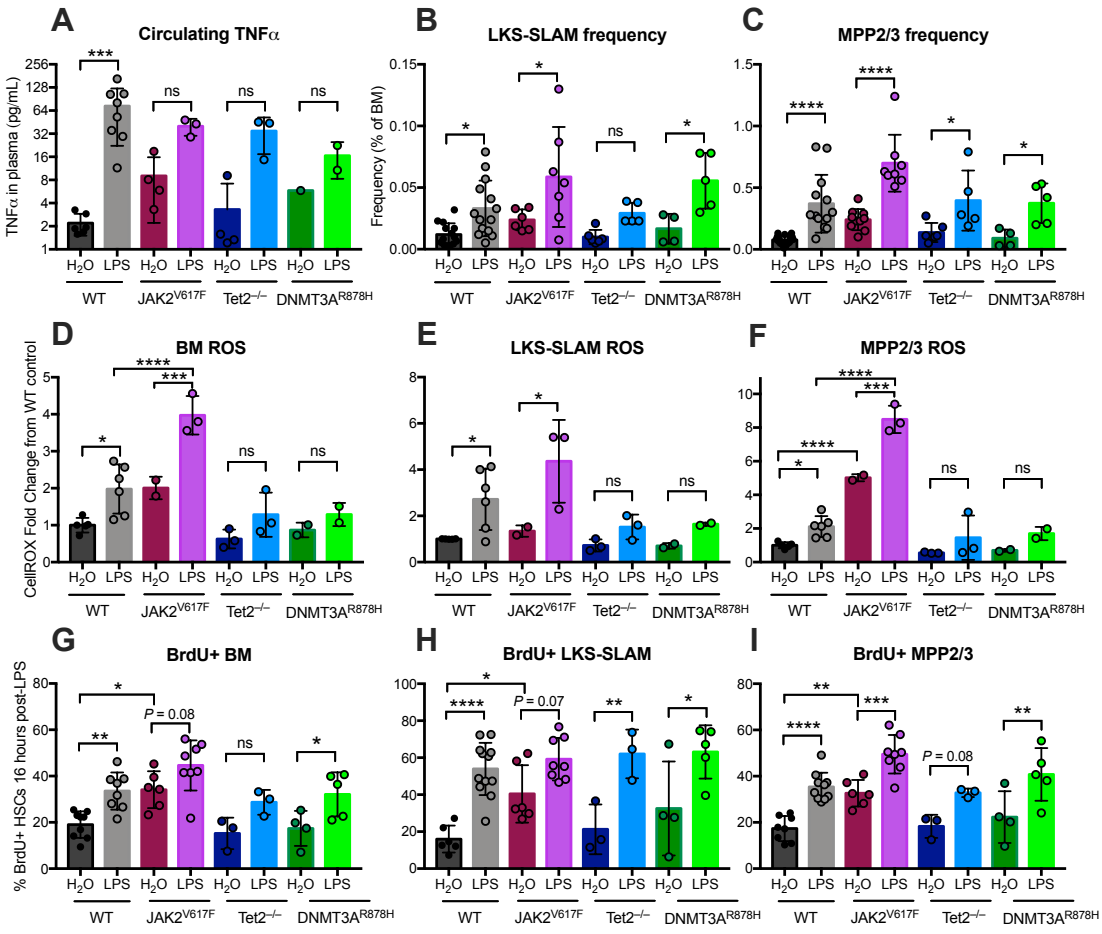
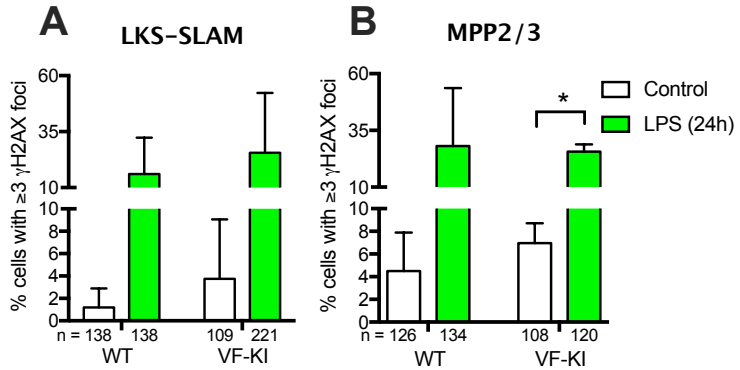
Supplemental



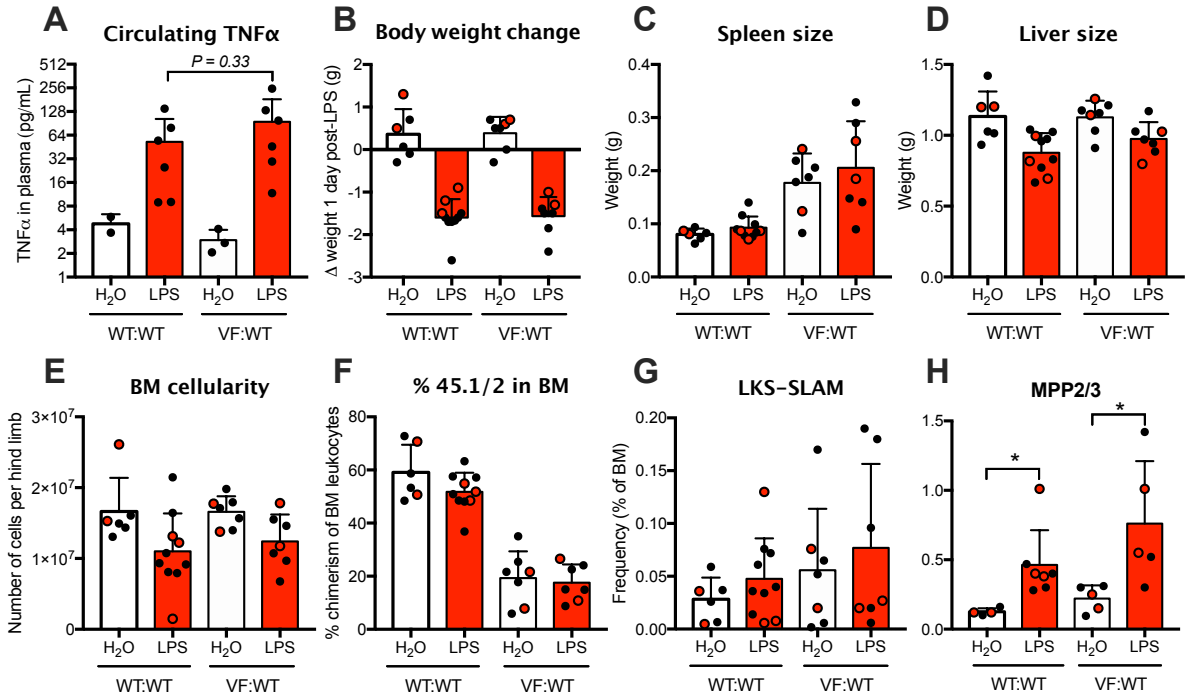
Supplemental Figure 2.1. Time course of normal physiological response following acute inflammation. A. Change in body weight (grams) following acute LPS treatment (0.5mg/kg). B-C. Weights of (B) spleens and (C) livers. D. Mean side scatter of total bone marrow cells. E-H. Peripheral blood (E) white blood cells, (F) platelets, (G) lymphocytes, (H) granulocytes. I-K. Absolute number of (I) LKS, (J) LKS-SLAM and (K) MPP2/3 cells per leg. Error bars represent SD, n=4-8/group; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; 2way ANOVA.



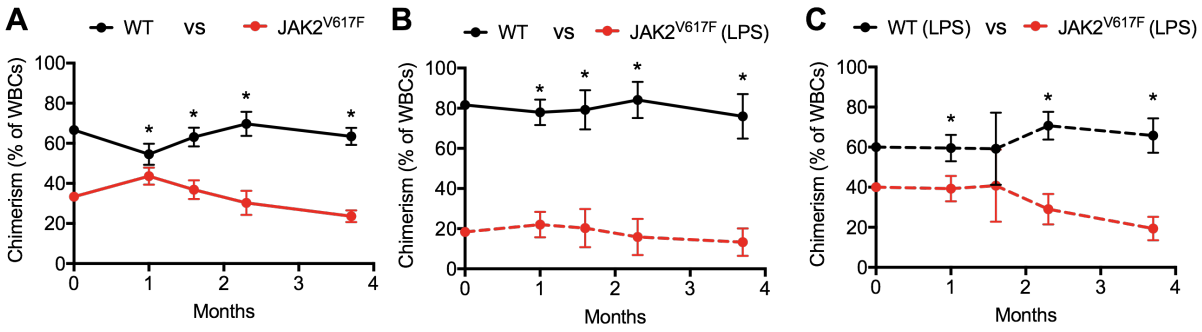
Supplemental Figure 2.2. Normal versus CH-mutant hematopoietic stem cell and progenitor frequency, oxidative stress and cycling following acute inflammation. A. TNF ELISA from mouse plasma. B-C. Frequency of (B) LKS-SLAM and (C) MPP2/3 of total BM cells. D-F. Fold change of CellROX mean fluorescence intensity normalized to untreated control in (D) BM, (E), LKS-SLAM, and (F) MPP2/3 cells. G-I. % of cells with BrdU incorporation in (G) BM, (H) LKS-SLAM and (I) MPP2/3 cells. Error bars represent SD, n=2-8/group; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; one-way ANOVA.



Supplemental Figure 2.3. Quantification of γH2AX immunofluorescence following LPS-induced inflammation. Frequency of cells with 3 or greater number of γH2AX foci in sorted (A) LKS-SLAM or (B) MPP2/3 cells. Error bars represent SD, n=2-4/group; *P<0.05, t-test.



Supplemental Figure 2.4. Physiological response to acute inflammation in WT:WT versus VF:WT transplanted mice. A. TNF ELISA from mouse plasma collected 16h post-LPS treatment. B. Change in body weight (grams). C-D. Weights of (C) spleens and (D) livers. E. Number of bone marrow cells per leg. F. Frequency of CD45.1/2 WT or VF cells in BM. G-H. Frequency of (G) LKS-SLAM and (H) MPP2/3 +/– LPS treatment. Error bars represent SD, n=5-8/group; *P<0.05; 2way ANOVA. Red dots represent NAC treated mice.



Supplemental Figure 2.5. Chronic LPS impairs normal and JAK2 mutant HSC fitness. Peripheral blood chimerism of donor-derived cells where equal ratios of bone marrow cells were transplanted into lethally irradiated recipients. A. Both WT and JAK2^{V617F} donors were untreated. B. Only JAK2^{V617F} donor mice were chronically treated with LPS. C. Both WT and JAK2^{V617F} donors were chronically treated with LPS (10μg/day for 30 days). n=3-5/group, error bars represent SEM, *P<0.05, **P<0.01; unpaired t-test.

CHAPTER 3

Thrombosis is a major cause of death in MPN patients so a key therapeutic strategy in MPN is to reduce thrombotic risk. Our JAK2^{V617F} knock-in mouse model is a unique MPN model because these mice die at an early age presumably from a thrombotic event. We unexpectedly found that the antioxidant NAC extends lifespan of the knock-in mice, so this chapter aims at identifying the mechanism of thrombosis inhibition with NAC treatment. First, we confirmed that NAC inhibits thrombosis in vivo using a chemically induced pulmonary thrombosis assay where collagen and epinephrine are injected intravenously to rapidly induce platelet activation, aggregation and subsequent thrombosis. Given that platelets are the major players in thrombosis development, we first focused on effects of NAC on platelet activation. Interestingly, NAC had no effect on platelet activation unlike aspirin, the current standard of care which permanently inactivates platelet activation.

Thrombosis can be induced by cells other than platelets, such as by granulocytes during the formation of neutrophil extracellular traps (NETs). Wolach et al suggested that MPN patients have elevated levels of NETs which contribute to thrombosis generation. Indeed, JAK2^{V617F} knock-in mice have elevated granulocyte counts and hypoactive platelets compared to wildtype mice so it's plausible that these mice die at an early age from NET-induced thrombosis and that NAC inhibits thrombosis in vivo via inhibition of NET formation. Here, we performed thrombosis assays in vivo and biochemical assays in vitro using murine and human blood to investigate the mechanisms of thrombosis independent of platelet activation.

Article

N-acetylcysteine inhibits thrombosis in a murine model of myeloproliferative neoplasm

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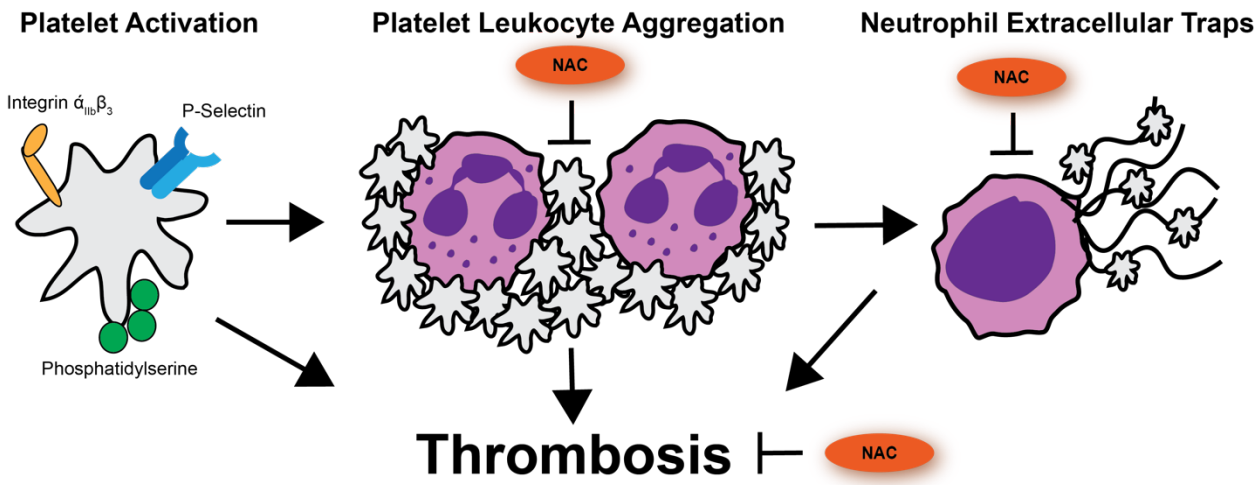
Key Points

1 The antioxidant N-acetylcysteine extends survival of the *Vav*-Cre:JAK2^{V617F} knock-in murine model of polycythemia vera.

2 N-acetylcysteine has the potential to inhibit thrombosis caused by the JAK2^{V617F} mutation.

Visual Abstract



Abstract

Thrombosis is a major cause of mortality in patients with myeloproliferative neoplasms (MPNs), though there is currently little to offer MPN patients beyond aspirin and cytoreductive therapies such as hydroxyurea for primary prevention. Thrombogenesis in MPN involves multiple cellular mechanisms, including platelet activation and neutrophil-extracellular trap (NET)

formation; therefore, an anti-thrombotic agent that targets one or more of these processes would be of therapeutic benefit in MPN. Here, we treated the JAK2^{V617F} knock-in mouse model of polycythemia vera with N-acetylcysteine, a sulfhydryl-containing compound with broad effects on glutathione replenishment, free radical scavenging and reducing disulfide bonds, to investigate its anti-thrombotic effects in the context of MPN. Strikingly, NAC treatment extended the lifespan of JAK2^{V617F} mice without impacting blood counts or splenomegaly. Using an acute pulmonary thrombosis model *in vivo*, we found that NAC reduced thrombus formation to a similar extent as the irreversible platelet inhibitor aspirin. *In vitro* analysis of platelet activation revealed that NAC reduced thrombin-induced platelet-leukocyte aggregate formation in JAK2^{V617F} mice. Furthermore, NAC reduced neutrophil extracellular trap formation in primary human neutrophils from MPN patients as well as healthy controls. These results provide evidence that N-acetylcysteine inhibits thrombosis in JAK2^{V617F} mice and provide a pre-clinical rationale for investigating NAC as a therapeutic to reduce thrombotic risk in MPN.

Introduction

Myeloproliferative neoplasms (MPN) are a group of chronic hematologic malignancies that include polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) and are characterized by the over-production of mature myeloid cells. The constitutive activating mutation in the Janus kinase 2 (i.e. JAK2^{V617F}) is the most common mutation found in MPN patients. Individuals harboring JAK2^{V617F} mutations are at high risk for thrombosis, which is a major cause of morbidity and mortality in patients with MPN. Thus, the primary goal of treatment in PV and ET is to reduce thrombotic risk. The current arsenal of agents to reduce thrombotic risk in MPN includes aspirin and cytoreductive agents such as hydroxyurea. Aspirin is an irreversible platelet inhibitor, current National Comprehensive Cancer Center Network (NCCN) guidelines

recommend low dose aspirin (81-100mg daily) for all JAK2 mutated MPN patients who are not at high risk of bleeding due to acquired von Willebrand disease[1]. Although aspirin has been established to reduce the risk of thrombotic events in PV patients[2], in low-risk ET patients (JAK2 negative and without cardiovascular risk factors) antiplatelet therapy with aspirin does not reduce the incidence of thrombotic events[3]. Cytoreductive agents such as hydroxyurea or anagrelide are given to high-risk patients (age >60 or previous thrombosis) to reduce thrombotic risk. However, in ET patients aged 40 to 59 years and lacking high-risk factors for thrombosis or extreme thrombocytosis, preemptive addition of hydroxyurea to aspirin does not reduce vascular events[4]. Moreover, cytoreductive drugs are not without significant side effects including GI toxicities, edema, palpitations, skin ulcers, increased risk of skin cancer, and potentially promotion of leukemia. Thus, treatments that reduce thrombotic risk in MPN without significant side effects would improve the quality of life and outcome in MPN patients, particularly in younger MPN patients.

Since the discovery of the JAK2^{V617F} mutation in 2005[5-9], numerous mouse models have been developed to study MPN *in vivo*[10]. The conditional *Vav*-Cre:JAK2^{V617F} knock-in mouse model of MPN die at approximately three months of age, presumed to be from thrombosis given that death is sudden without evident preceding decline in health[11]. These mice have elevated blood counts and splenomegaly but do not develop bone marrow fibrosis. Thus, this JAK2^{V617F} knock-in mouse model is ideal to identify therapeutics that can be used to prevent thrombosis in MPN.

Multiple cell types including platelets, leukocytes and endothelial cells participate in the formation of a blood clot. Damaged endothelium exposes collagen to platelets, leading to their activation and formation of a plug. There is a growing appreciation for the crucial role of leukocytes in thrombus initiation and progression, as platelet activation can cause activation of

leukocytes (most commonly of neutrophils), leading to formation of platelet-leukocyte aggregates (PLAs). A growing body of evidence supports the notion that aberrant leukocyte activation plays an important role in thrombosis in MPN[12]. Interestingly, an elevated leukocyte count, but not platelet count[13], correlates with thrombotic risk and cardiovascular disease[14] in MPN. Moreover, elevated levels of circulating PLAs are correlated with thrombotic risk in MPN[15].

Formation of PLAs can induce neutrophil extracellular trap (NET) formation (NETosis), a phenomenon dependent on oxidative stress whereby neutrophils release chromatin lined with granular components with antimicrobial properties. The primary function of NETs is to entrap pathogens for host defense, but they can also bind platelets and red blood cells contributing to thrombosis. Neutrophils from MPN mouse models as well as MPN patients are primed for NETosis[12], implicating involvement of NETs in the pro-thrombotic phenotype of MPN. Thus, preventing leukocyte activation and NETosis in MPN may be an effective approach to reduce thrombosis. Agents which not only act as reactive oxygen species (ROS) scavengers but also have anti-thrombotic properties with a favorable safety profile/minimal side effects would be attractive therapeutics for the prevention of thrombosis in MPN.

N-acetylcysteine (NAC) is an agent that has antioxidant, mucolytic and anti-inflammatory properties. It is currently used for acetaminophen overdose, contrast nephropathy prophylaxis, and as a mucolytic agent in cystic fibrosis. It has proven safe even at the large doses of greater than 10 grams given for acetaminophen overdose. NAC is being investigated as a therapy for thrombotic diseases such as Thrombotic Thrombocytopenic Purpura (TTP) (NCT01808521). Given that NETosis is dependent on oxidative stress, NAC has been found to block NETosis in vitro[16]. Therefore, NAC may prevent NETosis in MPN leading to reduced thrombosis. NAC has also been shown to be thrombolytic in arterial thrombi[17] and to reduce the formation of platelet-leukocyte

aggregates in a model of experimental diabetes[18]. In addition, NAC has anti-inflammatory properties and decreases serum inflammatory cytokines[19] which would also be beneficial in MPN. To that end, we tested the efficacy of oral treatment with NAC in JAK2^{V617F} knock-in mice with respect to their lifespan, thrombosis, and parameters such as blood counts, splenomegaly and tissue pathologies. We found that NAC reduces thrombosis in these mice via inhibition of PLA and NET formation. Therefore, NAC may represent a low-risk therapy for the reduction of thrombosis in MPN and should be investigated in human studies.

Materials and Methods

Mice

All animal procedures were performed under approval of the Institutional Animal Care and Use Committee at the University of California, Irvine. Equal numbers of male and female mice were used for all experiments. We used the *Vav-Cre:JAK2^{V617F}* murine model that has been previously described[11]. Mice were given drinking water with added N-acetylcysteine (2g L^{-1}) or aspirin (16mg L^{-1}) beginning at 4-6 weeks of age and replenished weekly. This dose of aspirin (16mg/L) translates to 3.2mg/kg/day assuming the average mice weighs 20g and drinks 4 ml of water per day. Since aspirin is used in a wide range of doses ranging from 0.15mg/kg to 60mg/kg in mice, we chose a low-dose aspirin treatment since MPN patients are prone to both thrombotic and hemorrhagic events. Complete blood counts were measured using the ABCVet Hemalyzer (scil) using blood diluted 1:1 with 100mM EDTA. Spleen and liver weights were recorded upon sacrifice or death.

Acute Pulmonary Thrombosis Model

An *in vivo* acute pulmonary thrombosis model was performed as described previously[20]. Briefly, mice were anesthetized with isoflurane and were then intravenously injected with a

mixture of collagen (0.8mg/kg) and epinephrine (6mg/kg). Circulating platelet counts were recorded using the ABCVet Hemalyzer 1 hour before and 5 minutes after treatment with collagen/epinephrine. Survival of mice was recorded as 30 minutes post-treatment. Immediately following death (or 30 minutes post-treatment), the lungs were perfused with phosphate-buffered saline and fixed in 10% zinc formalin for histological processing. At least 3 non-overlapping images per lung section were taken using the BZ-X700 Keyence brightfield microscope. Thrombi were measured and quantified by an investigator blinded to the conditions.

Platelet-Leukocyte Aggregates

All experiments involving human blood were performed under IRB approval from the University of California, Irvine. We used whole blood flow cytometry to detect platelet-leukocyte aggregates (PLAs) using fluorochrome-conjugated monoclonal antibodies to identify platelets and leukocytes. Briefly, 5 µl of whole blood was diluted in 55 µl of modified HEPES buffer without calcium (145mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.5mM $NaHPO_4$, 5mM glucose, 10mM HEPES-sodium) within 30 minutes of collection. Diluted blood was gently mixed with an antibody mix containing 0.2 µg CD41-APC (MWReg30, ThermoFisher Scientific), 0.2 µg CD45-PerCP-Cy5.5 (30-F11, BioLegend) and incubated for 20 minutes at room temperature, protected from light. To measure PLA subtypes, blood was also incubated with Alexa Fluor 488-Ly6G (1A8, BioLegend) and PE-CD11b (M1/70, BioLegend). For agonist-induced and inhibition experiments, thrombin at 1U/ml (Millipore Sigma). 1xRBC lyse/fix solution (BioLegend) was then added and further incubated for 15 minutes. Acquisition was performed within 30 minutes after RBC lysis. The percentage of $CD45^+/CD41^+$ double positive events representing PLAs was recorded.

***In vitro* NET formation**

To assess the effect of NAC on neutrophil extracellular trap formation *in vitro,* neutrophils were isolated using the EasySep Direct Human Neutrophil Isolation Kit according to the vendor's protocol (StemCell Tech, Vancouver, Canada). Neutrophils were resuspended in RPMI 1640 containing 2% fetal bovine serum (Life Technologies) then were seeded at 2×10^5 cells/chamber on Permanox plastic chamber slides coated with Poly-L-Lysine (Thermo Fisher, Rochester NY). Neutrophils were incubated at 37°C with 1mM N-acetylcysteine for 30 min before stimulation with 10nM Phorbol 12-myristate 13-acetate (PMA) for 3-6 hours at 37°C. Cells were fixed with 2% paraformaldehyde and incubated overnight at 4°C. Immunofluorescence was performed to detect the NET marker citrullinated histone 3 as previously described[12]. Briefly, cells were washed 3X with PBS, permeabilized with 0.1% Triton X-100 + 0.1% sodium citrate for 10 minutes at 37°C then blocked with 3% bovine serum albumin for 90 min at 37°C. Cells were incubated with 1:1000 rabbit anti-histone 3 citrullinated overnight at 4°C then with 1:2000 of Alexa 488 donkey anti-rabbit IgG for 2 hours at 25°C (ab5103 and ab150073, respectively; Abcam). DNA was counterstained with DAPI then slides were cover-slipped with Permafluor mountant (Thermo Fisher). Micrographs were taken with the BZ-X700 Keyence fluorescence microscope. NETs were quantified based on $H3^{cit}$ positivity or by swollen/delobular nuceli or extracellular DNA web-like strands. Percentages of NETs were determined based on 3-5 images per condition or >50 neutrophils per condition. NETs were quantified by 2 investigators blinded to the conditions.

SYTOX-based detection of extracellular DNA: We seeded 1×10^5 purified neutrophils/well into a cell imaging 96-well plate (Eppendorf) then added 1 μM SYTOX Green nucleic acid stain (Life Technologies). Cells were pre-treated and stimulated similar to immunofluorescence assays. Plates were incubated at 37°C, 5% CO2 in a Biotek Cytation 5 imaging plate reader, and fluorescence

was measured every 15 min at 504/523 nm for up to 16h. Neutrophil extracellular traps were measured by fluorescence as a result of extracellular DNA release.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism version 7.0 (Graphpad, La Jolla, CA, USA). For survival analyses as represented by Kaplan-Meier curves, a Log-rank (Mantel-Cox) test was performed. Other statistical analyses were performed using a one-way ANOVA (Sidak's multiple comparison test) or 2way ANOVA (Tukey's test). Significant differences were indicated as *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001. All experiments were performed at least three independent times unless otherwise stated.

Results

N-acetylcysteine extends the lifespan of JAK2^{V617F} knock-in mice

To investigate the effect of NAC on MPN pathology, in particular thrombosis, we added NAC (2g/L) or aspirin (16mg/L) to the drinking water of JAK2^{V617F} knock-in mice at 2 months of age and maintained them on this water continuously thereafter. Consistent with previous reports, JAK2^{V617F} knock-in mice begin to die at around 3 months of age without signs of preceding illness, consistent with thrombosis as the cause of death[11]. In support of this, we observed vascular occlusions in the lungs of JAK2^{V617F} but not WT mice (Figure S3.1). Strikingly, treatment with NAC extended the lifespan of *JAK2^{V617F}* mice past 12 months of age (Figure 3.1A). Aspirin, however, did not increase the lifespan of these mice (Figure 3.1A). N-acetylcysteine had no effect on spleen or liver weights (Figure 3.1B-C) nor on spleen, liver, or bone marrow architecture (Figure S3.1). In addition, NAC had no effect on blood counts in wild-type nor JAK2^{V617F} knock-in mice (Figure 3.1D-F). This data suggests that NAC extends the lifespan of mice with MPN

through a mechanism independent of cytoreduction. Based on this data, we postured that NAC may be inhibiting thrombus formation in *JAK2*^{V617F} knock-in mice.

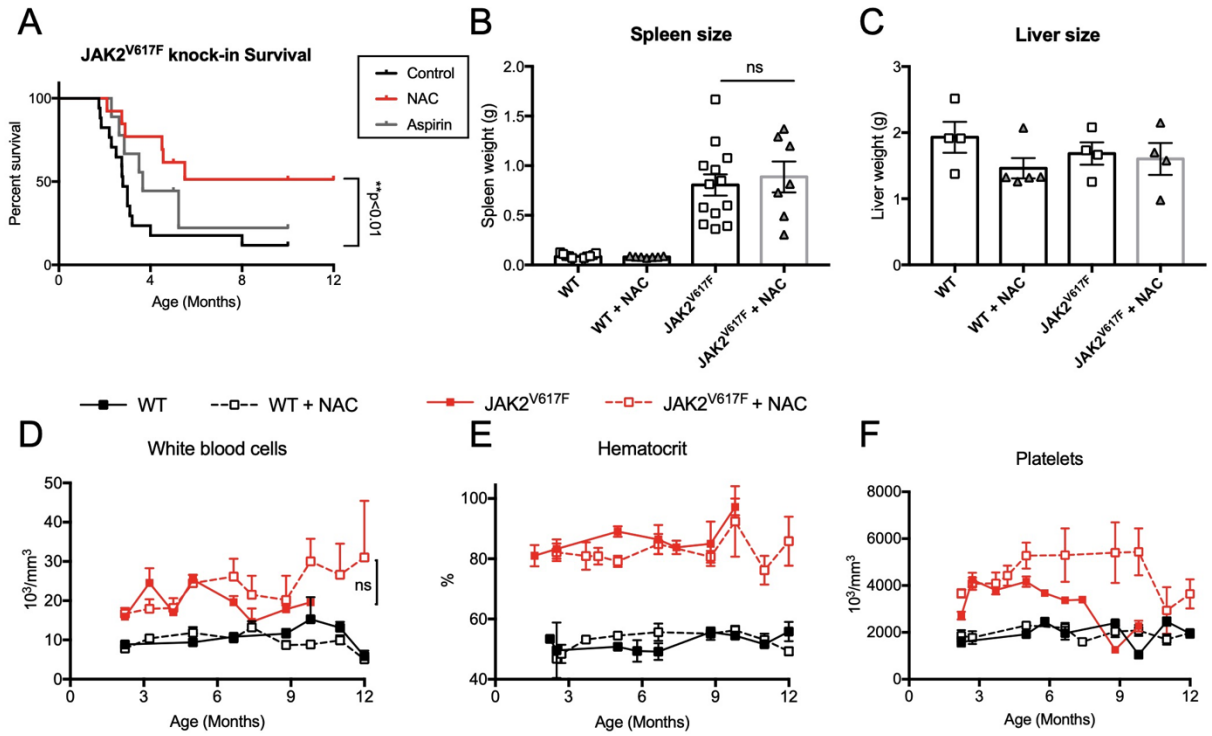


Figure 3.1. N-acetylcysteine (NAC) extends the lifespan in a JAK2^{V617F} knock-in mouse model of MPN. (A) Kaplan-Meyer survival curve representing *JAK2*^{V617F} mice whose drinking water contained NAC (2g/L) or aspirin (16mg/L), ***P*<0.01 Log-Rank test. (B) Spleen and (C) liver weights at sacrifice or death. (D-F) Peripheral blood counts, including (D) white blood cell count, (E) hematocrit and (F) platelet count in wild-type (WT) or *JAK2*^{V617F} mice treated with plain water or NAC-containing water. Error bars represent SEM, n=6-12/group. Representative data from two biological replicates.

N-acetylcysteine inhibits thrombus formation in vivo

To investigate the effect of NAC on thrombosis, we utilized an acute pulmonary thrombosis model as previously described[20]. This model involves activating platelets *in vivo* using a mixture of collagen and epinephrine then recording survival and histologically analyzing lung tissues for thrombi. Circulating platelet counts were measured immediately before and 5 minutes following collagen/epinephrine injection (Figure 3.2A). As expected, the number of circulating platelets was significantly reduced in mice treated with collagen/epinephrine compared to

untreated controls ($p<0.001$, 2way ANOVA), suggesting a thrombus formation in the lungs (Figure 3.2A). Aspirin irreversibly inhibits platelet activation and was used as a positive control in this assay. As expected, aspirin-treated mice had significantly higher circulating platelets compared to PBS-treated mice following collagen/epinephrine treatment ($p<0.01$, 2way ANOVA; Figure 3.2A). Strikingly, NAC-treated mice phenocopied aspirin-treated mice with a significantly higher circulating platelet count following collagen/epinephrine treatment compared to PBS controls ($p<0.01$, 2way ANOVA; Figure 3.2A). All mice (6 of 6) treated with collagen/epinephrine alone died within 30 minutes post-injection as a result of the pulmonary thrombi formation (Figure 3.2B). However, 50% of NAC-treated mice (3 of 6) survived collagen/epinephrine treatment while 33% of aspirin-treated mice (2 of 6) survived collagen/epinephrine treatment (Figure 3.2B). Upon histological analysis of the lungs, pulmonary thrombi were clearly visible in mice treated with the collagen/epinephrine mixture, with an average of 4 thrombi per field (Figure 3.2C, E). There were significantly fewer visible thrombi in NAC-treated mice (1.5 thrombi/field; $p<0.01$, 2way ANOVA) as well as aspirin-treated mice (1.6 thrombi/field; $p<0.01$, 2way ANOVA) compared to collagen/epinephrine treatment alone (Figure 3.2C, F and G). These data indicate that NAC inhibits thrombus formation induced by collagen and epinephrine-induced platelet activation as well as aspirin *in vivo.*

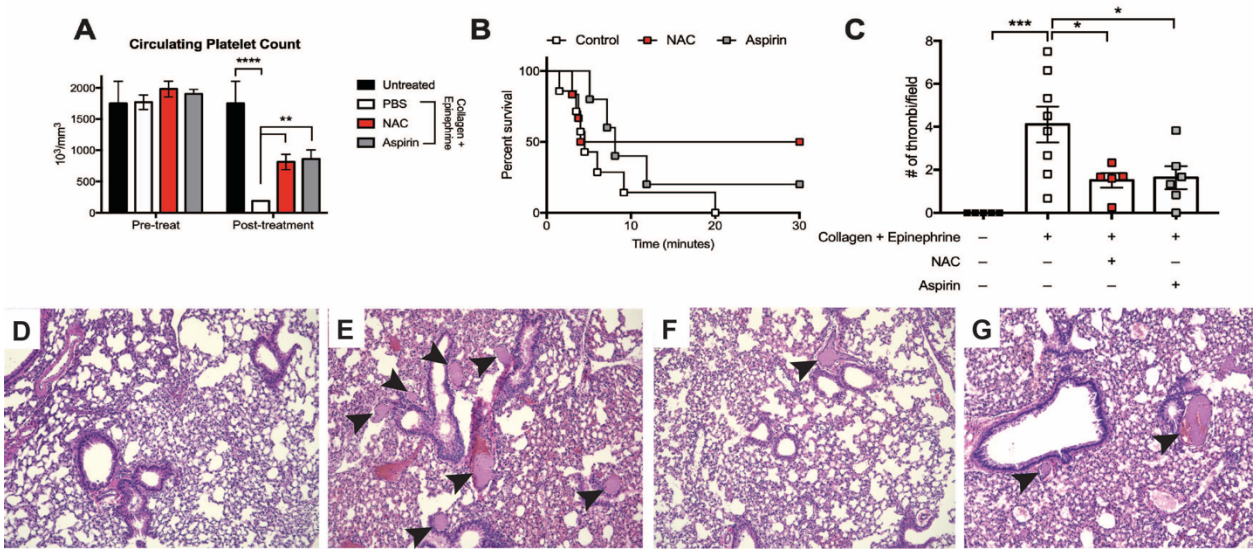


Figure 3.2. N-acetylcysteine reduces pulmonary thrombus formation *in vivo*. (A) Platelet counts in adult mice 1 hour before and 5 minutes following intravenous injection with a mixture of collagen (0.8mg/kg) + epinephrine (6mg/kg). (B) Kaplan-Meyer curve representing survival of mice injected with collagen + epinephrine mixture to induce a thromboembolism. Survival was recorded as 30 minutes post-injection. (C) Quantification of pulmonary thrombi per image field. (D-G) Representative histology images of lungs from (D) untreated control, (E) collagen/epinephrine-treated, (F) NAC + collagen/epinephrine, or (G) aspirin + collagen/epinephrine. Pulmonary thrombi are indicated by black arrowheads, n=6 mice/group, 20× magnification. ***P*<0.01, ****P*<0.001, *****P*<0.0001, 2way ANOVA. Error bars represent SEM. Representative data from three biological replicates.

N-acetylcysteine rescues mice containing JAK2^{V617F} cells from an induced thrombosis

Given that *Vav*-Cre expression can be found in endothelial cells, we utilized a transplantation approach to interrogate how JAK2^{V617F} expression in hematopoietic cells alone influences thrombogenesis and the impact of NAC on this. We transplanted equal numbers of JAK2^{V617F} and wildtype bone marrow cells into lethally irradiated wildtype recipients and performed acute pulmonary thrombosis assays 3 months post-transplant. Compared to mice transplanted with wildtype cells alone (WT:WT), mice containing JAK2^{V617F} cells (VF:WT) were more susceptible to induced pulmonary thrombosis in response to thrombotic agents (Figure 3.3). All VF:KI mice (6 of 6) treated with 1× dose or ½× dose of collagen/epinephrine alone died within 30 minutes while 25% (1 of 4) of WT:WT mice survived (Figure 3.3A, B). At ¼× dose of collagen/epinephrine, 83% (5 of 6) WT:WT mice survived while only 33% (1 of 3) of VF:WT

mice survived (Figure 3.3C). These data suggest that mice containing JAK2^{V617F} hematopoietic cells are hypersensitive to an induced thrombosis. Similar with wildtype mice, we observed that NAC significantly prolonged survival of VF:WT mice following induction of thrombosis with collagen/epinephrine (Figure 3.3A). While 100% of VF:WT mice ultimately succumbed to death from collagen/epinephrine at full dose, NAC prolonged survival from 3.75 minutes post-induction in controls to over 14 minutes post-induction with NAC (P=0.01, Log-rank test; Figure 3.3A). NAC also increased the percentage of VF:WT mice surviving to 50% with the ½× dose of collagen/epinephrine (Figure 3.3B). Concurrent with the survival trend, we observed fewer thrombi in lung tissues of NAC and aspirin-treated VF:WT mice compared to controls (Figure 3.3D-G).

As platelet activation is a key component of thrombosis, we first investigated whether NAC may prevent thrombosis by inhibiting platelet activation. Platelets from JAK2^{V617F} mice did not show basal hyperactivation as measured by P-selectin exposure, αIIbβ3 integrin activation or phosphatidylserine (PS) exposure and also did not show increased agonist-induced activation as compared to platelets from wild-type mice (Figure S3.2). NAC treatment had no marked effect on agonist-induced integrin activation and P-selectin (CD62P) exposure on wild-type nor JAK2^{V617F} platelets (Figure S3.2). NAC had no effect on ionomycin-stimulated PS exposure in wild type nor mutant platelets (Figure S3.3). These observations suggest that spontaneous and fatal thrombus formation in JAK2^{V617F} knock-in mice is not a result of a hyperactive platelet phenotype and that NAC has no marked effect on platelet activation responses.

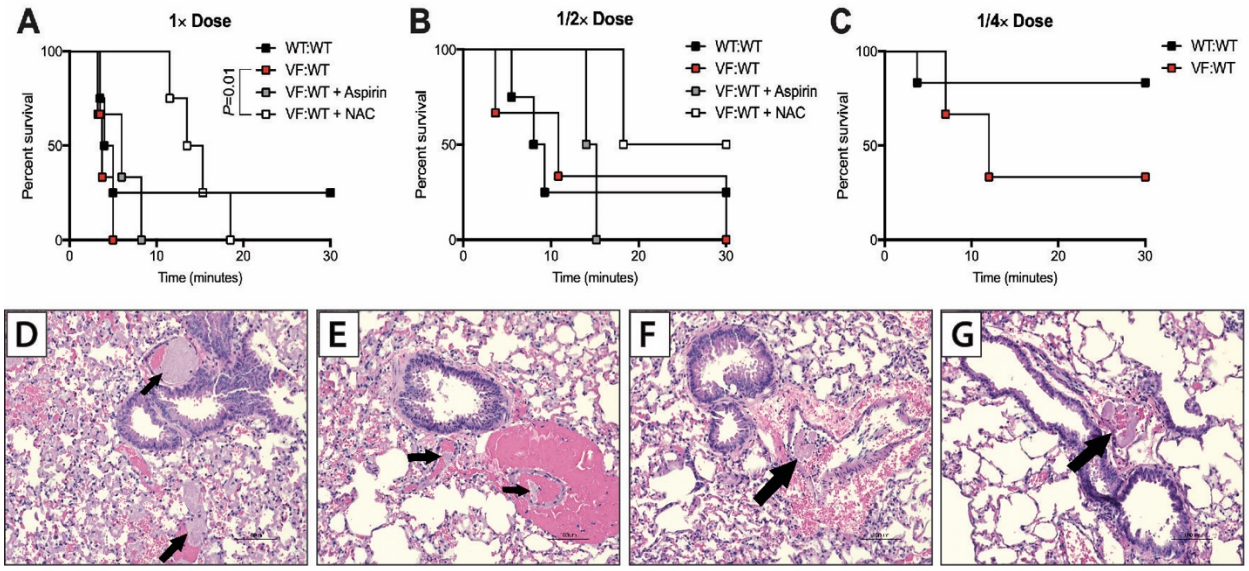


Figure 3.3. NAC improves survival following induction of acute thrombosis in mice transplanted with JAK2^{V617F} cells. Control (WT:WT) and JAK2^{V617F} engrafted (VF:WT) mice were pre-treated with aspirin (50mg/kg) or NAC (400mg/kg) for 1 hour and then treated with decreasing concentrations of a mixture of collagen and epinephrine via retro-orbital injection. Kaplan-Meyer curve representing survival of mice injected with collagen and epinephrine concentrations of (A) 0.8mg/kg and 6mg/kg, (B) 0.4mg/kg and 3mg/kg and (C) 0.2mg/kg and 1.5mg/kg, respectively. (D-G) Representative H&E-stained lung tissues from collagen and epinephrine-injected mice. Treatments include (D) Control (WT:WT), (B) VF:WT, (C) VF:WT + NAC, (D) VF:WT + Aspirin. Arrows represent thrombi. *P*=0.01, Log-rank test. n=3-4 mice/group, 20× magnification. Representative data from two biological replicates.

N-acetylcysteine inhibits platelet-leukocyte aggregate formation in vitro

High levels of circulating platelet-leukocyte aggregates (PLAs) are linked to thrombosis and inflammation. To investigate how NAC impacts PLA formation *in vitro*, whole blood obtained from mice on NAC or plain water was stimulated with thrombin and then PLAs and platelet-neutrophils aggregates (PNAs) were measured by flow cytometry (Figure 3.4A). PLAs were also observed in murine blood smears following stimulation with thrombin (indicated by red arrowhead, Figure 3.4B-C). We observed a significant increase in PLAs and PNAs following thrombin stimulation in both wild-type and JAK2^{V617F} knock-in mice on plain water (Figure 3.4D-E). In wild-type mice on NAC water, thrombin stimulation resulted in a significant increase in PLA and PNA formation ($p<0.05$; Figure 3.4D-E). However, in JAK2^{V617F} mice on NAC water, there was no significant increase in PLA and PNA formation following thrombin stimulation

(Figure 3.4D-E), suggesting that NAC inhibits PLA formation in JAK2^{V617F} knock-in mice. Given that JAK2^{V617F} knock-in mice most closely resemble a PV phenotype, we measured circulating PLA and PNA levels in PV patients and normal controls. We observed a significant increase in circulating PLAs in PV patients (mean 9.0% +/– 1.1%, n=17) compared to normal controls (mean 5.2% +/– 0.7%, n=13; Figure 3.4F, p=0.00095, t-test). Similarly, we observed an increase in circulating PNAs in PV patients compared to normal controls, though this trend did not reach significance (Figure 3.4F-G, p=0.17, t-test). Taken together, these data suggest that NAC may reduce thrombosis and extend lifespan in JAK2^{V617F} knock-in mice in part through its ability to block platelet activation-induced PLA formation.

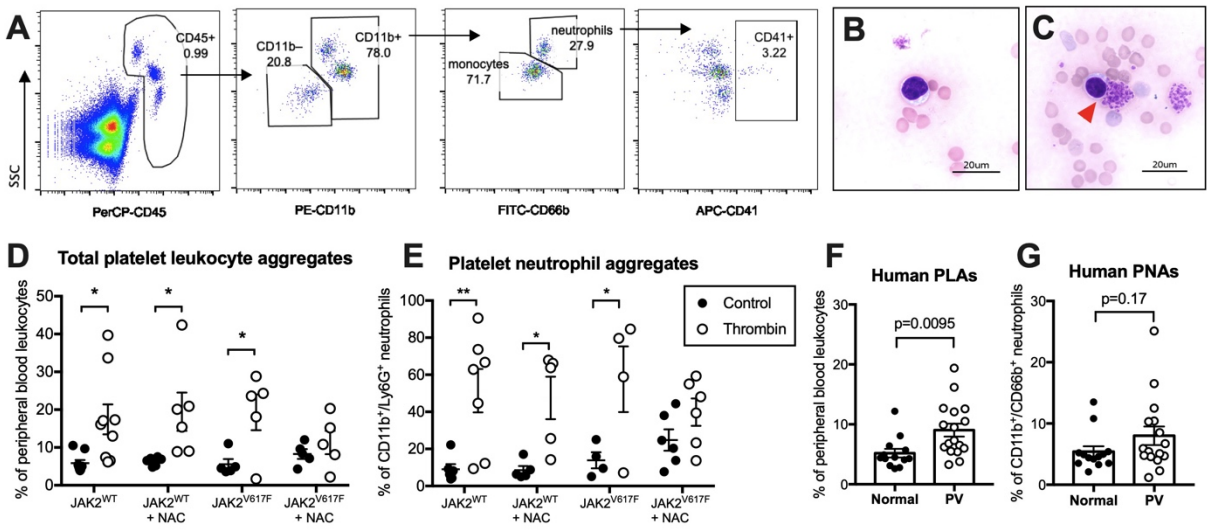


Figure 3.4. NAC inhibits thrombin-induced platelet leukocyte aggregate (PLA) formation in JAK2^{V617F} knock-in mice. (A) Representative gating strategy to measure circulating or thrombin-stimulated PLAs (based on CD45+/CD41+ double positive events). Platelet-neutrophil aggregates (PNAs) are defined as CD45+/CD11b+/CD66b+ (for human) or Ly6G+ (for mice)/CD41+ events. (B) Brightfield image of unstimulated murine blood smear stained with Wright Giemsa. (C) Brightfield image of PLA (denoted by red arrowhead) from murine blood stimulated with 2U thrombin; 60× magnification, scale bars represent 20µm. (D) Quantification of PLAs or (E) PNAs in wild-type or JAK2^{V617F} knock-in mice with or without NAC treatment. *P<0.05, **P<0.01, 2way ANOVA. (F) Circulating PLAs or (G) PNAs in fresh human blood from Polycythemia vera (PV) patients and healthy donors. Each data point represents an individual. Error bars represent SEM. Representative data from three biological replicates.

N-acetylcysteine inhibits neutrophil extracellular traps in vitro

Activation of neutrophils results in NETosis and this phenomenon has been shown to promote thrombosis in MPN[12]. To investigate the role of NAC on NET formation, we first assessed NETosis *in vitro* in human neutrophils by immunofluorescence (Figure 3.5). We confirmed that phorbol myristate acetate (PMA) is a strong activator of neutrophils and rapidly induces NET formation in neutrophils from MPN patients and normal controls, as measured by citrullinated Histone 3 positivity and DNA morphology ($P<0.01$ and $P<0.0001$, respectively, one-way ANOVA; Figure 3.5A). NAC reduced PMA-induced NET formation in both normal and MPN neutrophils (Figure 3.5B-C). We also utilized a fluorescence-based assay to measure extracellular DNA stained with the nucleic acid dye, SYTOX as a measure for NETosis (Figure 3.5D-E). A representative time course of PMA-induced NETosis from stimulated healthy neutrophils is shown in Figure 3.5D. Quantification of SYTOX relative fluorescence units (RFU) were normalized to unstimulated healthy neutrophil fluorescence (Figure 3.5E). We found a significant increase in RFU following PMA stimulation in both normal and MPN neutrophils ($P<0.00001$ and $P<0.001$ respectively, t-test; Figure 3.5E). Pre-treatment with NAC resulted in a significant decrease in PMA-induced NETs in MPN neutrophils (Figure 3.5E). To assess the impact of platelets on NET formation, we co-incubated neutrophils with platelets that were activated with thrombin (Figure S3.5). Consistent with other reports that showed activated platelets induce NET formation [21,22], we observed a modest increase in NET formation in normal as well as MPN neutrophils upon co-incubation with activated normal platelets (Figure S3.5). MPN platelets, however, induced NET formation even without *ex vivo* activation with thrombin (Figure S3.5). These data suggest that MPN platelets may be more apt to bind to and activate neutrophils leading to NET formation which contributes to the prothrombotic state in MPN.

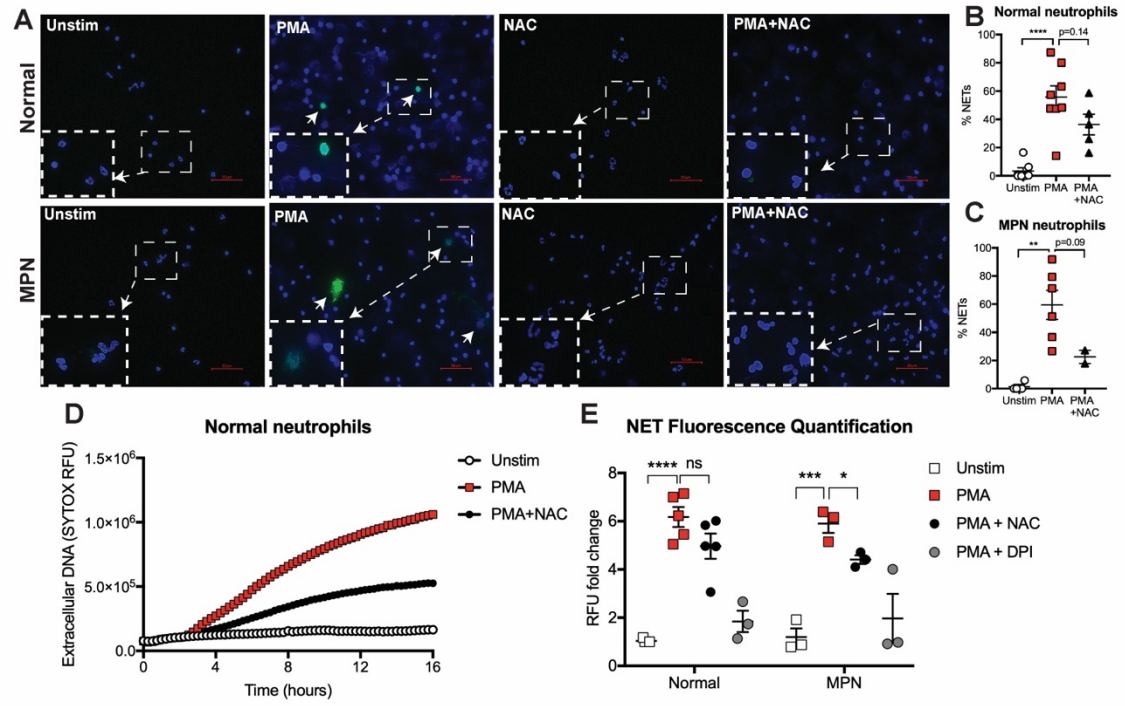


Figure 3.5. Neutrophil extracellular traps (NETs) are reduced by NAC in MPN patients. (A) Representative immunofluorescence images of NETs from normal and MPN individuals. Citrullinated H3 (H3cit) is shown in green and DAPI-stained DNA is shown in blue. Arrows represent H3cit^{+} NETs. Bars represent 50μm. Original magnification 40×. (B) Quantification of NETs from normal individuals or (C) MPN patients upon stimulation with 10nM PMA and PMA+NAC; **p<0.01, ****p<0.0001 one-way ANOVA. (D) Representative time course of extracellular DNA via relative fluorescence units (RFU) as measured by the nucleic acid dye SYTOX. (E) Quantification of RFU normalized to unstimulated normal neutrophils. The inhibitor diphenyleneiodonium (DPI) was used as a positive control for NET inhibition. Each point represents an individual; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 t-test. Error bars represent SEM. Representative data from three biological replicates.

Discussion

Here, we demonstrate that N-acetylcysteine extends lifespan in the *Vav*-Cre inducible JAK2^{V617F} knock-in mouse model of polycythemia vera. Consistent with previous reports, these mice have erythrocytosis, leukocytosis, and splenomegaly (Figure 3.1 and S3.1)[11,12]. We observed thrombocytosis in our mice, as would be expected for a PV phenotype, although an elevated platelet count was not observed in the original description of this model by Mullally et al[11]. Mice die suddenly without preceding illness and are found to have thrombi in the lungs at necropsy, implicating thrombosis as the cause of death in this model. However, we cannot overlook the possibility that these mice may also die from other causes, for example from hemorrhagic events,

which may explain why NAC did not rescue all JAK2^{V617F} mice. In addition, the *Vav-Cr*e inducible model does have expression of JAK2^{V617F} in endothelial cells, this study does not address the impact of N-AC specifically on endothelial cells. Nonetheless, our findings corroborate past work using the JAK2^{V617F} knock-in mouse model for anti-thrombotic studies and demonstrate for the first time an intervention that can extend survival of JAK2^{V617F} knock-in mice.

We found that NAC did not reduce blood counts or splenomegaly in JAK2^{V617F} knock-in mice, however Marty et al[23] previously reported that NAC reduced spleen weight, blood counts, and reduced the frequency of JAK2^{V617F} progenitors in a competitive transplantation JAK2^{V617F} murine model. The impact of NAC on blood counts and spleen size by Marty et al[23] could possibly be explained by the selective reduction of JAK2^{V617F} cells. Because hematopoiesis in our model is made up entirely of JAK2^{V617F} cells this could explain why we did not see the same impact on blood counts or spleen weight as Marty et al[23].

Using an acute pulmonary thrombosis model in wild-type mice as well as mice transplanted with JAK2^{V617F} hematopoietic cells, we found that NAC reduces thrombus formation *in vivo*. While NAC was as effective as aspirin at reducing an induced thrombus in wildtype mice, aspirin did not improve survival of JAK2^{V617F} knock-in mice, suggesting that NAC has additional benefits in MPN that are distinct from aspirin. Our data suggest that classical platelet activation is not the primary mechanism of thrombogenesis in this PV mouse model, and is in line with the lack of correlation between platelet count and thrombotic risk in MPN patients[3]. In fact, although platelet number and size were significantly elevated in the JAK2^{V617F} knock-in mice, this did not translate to a prothrombotic phenotype as platelet activation in response to agonists was unaltered by JAK2^{V617F} expression. Interestingly, the model with Mx1-Cre inducible human transgene of JAK2^{V617F} knock-in with an ET phenotype showed heightened platelet responses in vitro and

decreased tail bleeding time[24]. One possible explanation could be due to the different mouse models used. Moreover, another model of JAK2^{V617F} knock-in showed blunted platelet responses *in vitro* but rapid and unstable thrombus formation *in vivo*[25]. Similarly, platelet-specific (Pf4-Cre) expression of JAK2^{V617F} did not result in a pro-thrombotic phenotype while Tie2-Cre inducible JAK2^{V617F} caused dysfunctional hemostasis and carotid artery thrombosis[26]. Other groups using the same *Vav*-Cre inducible JAK2^{V617F} mouse model showed significantly increased predisposition for venous thrombosis in engrafted mice despite defective platelet responses *in vitro*[12]. We too observed increased mortality of JAK2^{V617F} engrafted mice in the pulmonary thrombosis model although platelet reactivity was unchanged.

We found that platelets from JAK2^{V617F} mice do not exhibit aberrant activation *in vitro* as assessed by conventional assays such as P-selectin, integrin, or phosphatidylserine exposure at baseline nor with stimulation. However, we found that platelets from MPN patients modestly induce NETs from both normal and MPN neutrophils without *ex vivo* stimulation, whereas normal control platelets only activate NETs with the addition of *ex vivo* stimulation. Our findings suggest that MPN platelets may play a crucial role in promoting NETosis in MPN, highlighting the complex interplay of these cells to induce NETosis.

In this study, we observed no marked difference in PMA-induced NETosis using purified neutrophils from MPN patients versus healthy controls. However, Wolach *et. al*. showed that MPN neutrophils exhibited elevated ionomcyin-induced NETosis[12]. Our results highlight the importance of stimulant type to induce differential NETosis between MPN and unaffected individuals. Certain types of NETosis, such as PMA-induced NETosis, are dependent upon ROS and are inhibited by NAC[27]. While oxidative stress has been linked to NETs, we did not observe elevated levels of reactive oxygen species at baseline or following stimulation in MPN patient leukocytes or in

JAK2^{V617F} knock-in mice model compared to healthy controls (Figure S3.4). Lower ROS levels that we have observed in MPN patient leukocytes could be attributed to an increased antioxidant potential in JAK2^{V617F} CD34+ progenitors[28].

Our study does not specifically address the impact of NAC on established thrombosis. Interestingly, NAC has been investigated for its thrombolytic effects in experimental models of acute ischemic stroke and was shown to promote lysis of arterial thrombi via reduction of disulfide bridges in large von Willebrand Factor (vWF) multimers leading to thrombus disintegration[17]. Even the JAK2^{V617F} mutation has been shown to induce proteolysis of vWF multimers[25] though we observe increased pulmonary thrombosis in mice engrafted with JAK2^{V617F} cells. Possible anti-thrombotic actions of N-acetylcysteine may be due the reduction of vWF multimers[29] as well its interactions with other clotting factors containing disulfide bonds. Moreover, NAC has been found to downregulate lysyl oxidase (LOX) activity through glutathione replenishment in idiopathic pulmonary fibrosis model[30]. Interestingly, overexpression of LOX has been identified in MPN and LOX overexpression in platelets leads to a pro-thrombotic phenotype that increases platelet adhesion to collagen[31].

In summary, this work identifies NAC as a potential agent to reduce thrombosis in MPN. The exact mechanism by which NAC rescues JAK2^{V617F} from thrombotic death requires further investigation though our findings suggest that NAC impacts the interaction between platelets and neutrophils in MPN. Given that NAC is a low-cost, widely available, safe agent our results encourage the evaluation of NAC in MPN patients for reduction of thrombotic risk. Thus, we are currently developing a clinical trial investigating the impact of NAC in MPN. Moreover, NAC may also prove to be beneficial in other clonal hematologic conditions such as clonal

hematopoiesis of indeterminate potential (CHIP)[32] which is associated with coronary heart disease as well as thrombosis.

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Authorship Contribution: B.M.C. performed experiments, analyzed the data, made the figures and wrote the manuscript; G.R. performed the experiments, designed experiments and wrote the manuscript; S.H. and E.C. performed platelet activation experiments; L.M.L performed neutrophil extracellular trap experiments; S.B and H.Y.L. discussed results and provided comments and feedback; A.G.F. planned, designed and coordinated the research and wrote the manuscript. All authors read the manuscript and approved its content.

Conflicts of Interest Disclosure: Incyte and Celgene Speakers Bureaus (AGF)

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Supplemental

Flow Cytometry to Assay Platelet Activation

Integrin GPIIb/IIIa and P-Selectin: Washed blood was prepared by diluting 50 µl of heparinized blood in Tyrode-HEPES buffer without calcium (134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 20mM HEPES, 5mM glucose, 1mM $MgCl_2$, 0.35% bovine serum albumin, pH 7.4) and centrifugation at 2800 rpm for 5 min at room temperature. The washing step was repeated and the cell pellet was resuspended in Tyrode-HEPES buffer containing 2 mM Ca^{2+}. Next, 50 µl of washed blood was added to FACS tubes containing antibody mix of CD41-APC (MWReg30, ThermoFisher Scientific), P-selectin-FITC (RB40.34, BD Biosciences), GPIIb/IIIa-PE (JON/A, Emfret Analytics, Germany) and thrombin (Millipore Sigma) at 0.05, 0.1 and 1 U/ml. The mean fluorescence intensity of P-selectin in $CD41^+$ platelets was recorded.

Phosphatidylserine Exposure: 20 µl of heparinized whole blood was diluted in 1:12 in saline (0.9% NaCl) and 80 µl of AnnexinV binding buffer [10 mM HEPES/NaOH, (pH7.4) 140 mM NaCl, 2.5 mM CaCl2] was added. Platelets were stimulated with agonists including collagen (3mg/ml) or thrombin (1U/ml). Diluted blood with or without stimulant was incubated for 10 minutes at 37°C. Whole blood was diluted further by mixing 10 µl of the suspension with 30 µl of Annexin V buffer. AnnexinV-PE (BioLegend) and FITC anti-mouse/rat CD61 (2C9.G2, BioLegend) were then added and incubated for 15 minutes at room temperature. Immediately before acquisition, 200 µl of AnnexinV binding buffer was added to the samples. The percentage of AnnexinV-positive, $CD61^+$ platelets was recorded.

Cellular oxidative stress

Reactive oxygen species (ROS) in murine PBMCs and neutrophils and was measured using dichlorofluorescein (DCF) fluorescence. Briefly, 30-50uL blood was collected via saphenous vein then red blood cells were lysed using ammonium-chloride-potassium lysing buffer. Cells were

washed 2X with PBS then stained with CD45-PerCP-Cy5.5 (30-F11, BioLegend), Alexa Fluor 488-Ly6G (1A8, BioLegend) and PE-CD11b (M1/70, BioLegend) and incubated for 15 minutes at 4°C, protected from light. Following washing, cells were resuspended in RPMI loaded with 5uM DCFH-DA (dichlorofluorescein diacetate; Life Technologies) for 30 minutes at 37°C in a 5% CO_2 incubator. Cells were then washed two times with PBS to remove excess dye followed by stimulation with phorbol myristate acetate (PMA) 10nM for 30 min. The mean fluorescence intensity of the DCF signal was recorded by flow cytometry using a BD Accuri C6 flow cytometer.

To measure ROS in human PBMCs, cells were isolated by density gradient centrifugation or fresh neutrophils were isolated using the EasySep direct human neutrophil isolation kit (STEMCELL Technologies, Vancouver, Canada). ROS were measured using the CellROX kit according to the vendor's instructions (Life Technologies). For Luminol ROS detection, $2x10^5$ isolated neutrophils in RPMI media were loaded with 500 μM Luminol (Sigma #123072) for 15 minutes at 37°C in a 5% CO_2 incubator. Cells were then stimulated with phorbol myristate acetate (PMA) 10nM or TNF-alpha 50ng/mL +/– 100 μM NAC. Luminescence kinetics were recorded using a Cytation5 Biotek plate reader. Quantification of ROS luminescence is recorded after 90 min incubation.

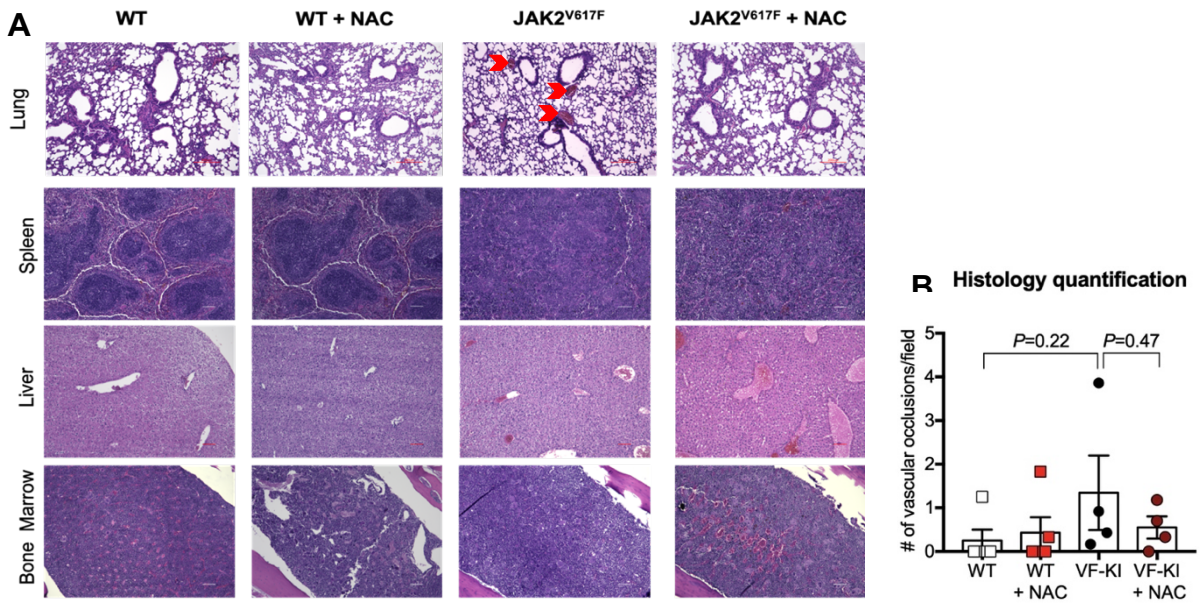

Figure S3.1. Tissue histology in wild-type (WT) and JAK2V617F mice +/– N-acetylcysteine (NAC) treatment. (A) Hematoxylin and eosin staining of lung, spleen, liver and bone marrow is represented. Red arrows represent vascular occlusions. Brightfield images were taken using a BZ-X700 Keyence brightfield microscope. Original magnification 10X. Scale bar represents 50um in spleen, liver and bone marrow images. Scale bar represents 200µm in lung histology images. (B) Quantification of vascular occlusions in lungs of WT and JAK2V617F (VF-KI) mice on +/– NAC. One way ANOVA.

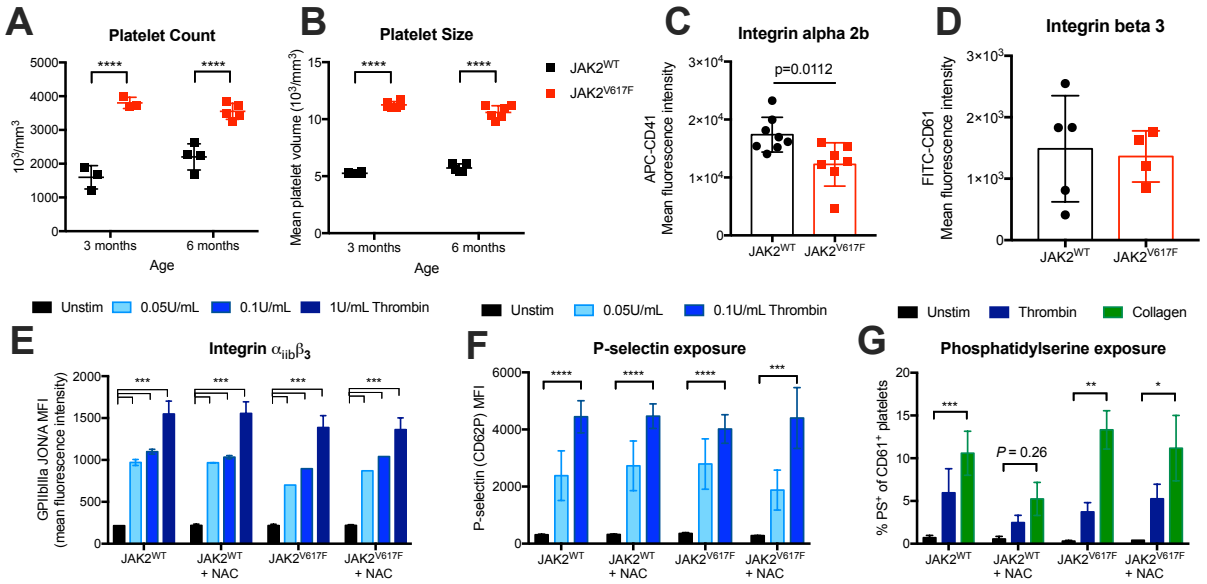

Figure S3.2. NAC does not alter JAK2^{V617F} platelet activation. (A) Total platelet count and (B) mean platelet volume in JAK2^{WT} or JAK2^{V617F}-knock in mice. (C) Integrin alpha 2b (CD41) and (D) Integrin beta 3 mean fluorescence intensity in platelets from wild-type and JAK2^{V617F}-knock in mice. (E) Integrin alpha2b/beta3 exposure on platelets following thrombin-stimulation. (F) P-selectin (CD62P) mean fluorescence intensity (MFI) following thrombin-stimulation. (G) Phosphatidylserine exposure on platelets following thrombin (1U/mL) or collagen (3mg/mL) stimulation. Data represents at least three independent experiments containing n=2-4 mice/experiment. Error bars represent SEM. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, 2way ANOVA.

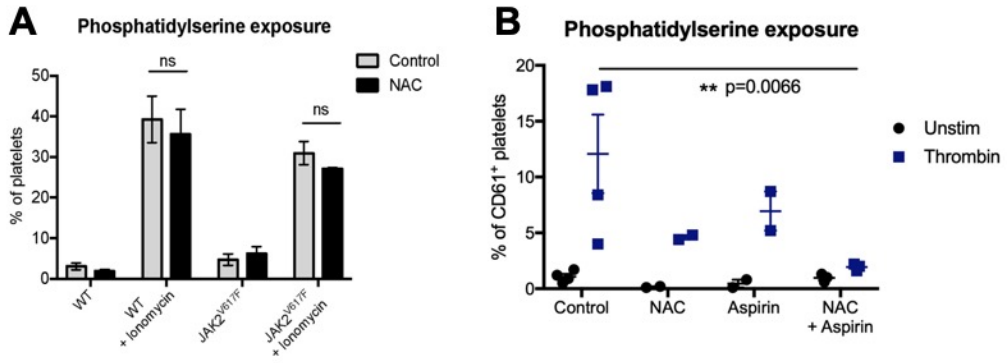

Figure S3.3. Platelet stimulant type influences effect of NAC phosphatidylserine (PS) exposure on platelets. (A) NAC has no effect on ionomycin-induced PS exposure in wild-type (WT) or JAK2V617F platelets (gated on CD61+ events), n = 3-6 mice/group. (B) Treatment with a combination of aspirin + NAC results in significantly reduced thrombin-induced PS exposure in WT platelets compared to untreated controls (**p=0.0066, 2way ANOVA), n=2-4/group. Error bars represent SEM. Representative data from three biological replicates.

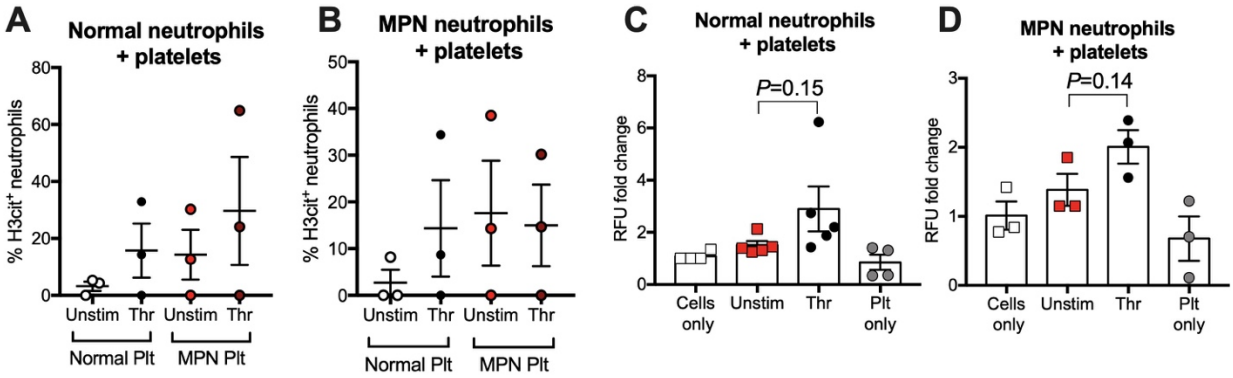

Figure S3.4. Activated platelets modestly induce NETosis. (A-B) Quantification of NETs as measured by $H3^{cit+}$ neutrophils from (A) healthy or (B) MPN individuals. (C-D) Fold change of relative fluorescence units from Sytox assay where results are normalized to fluorescence from normal neutrophils alone. Washed platelets were seeded at a 200:1 ratio to neutrophils. Thrombin (1U) stimulation induced modest yet insignificant increase in NETs from (C) normal and (D) MPN neutrophils, t-test $P>0.05$. Representative data from three biological replicates.

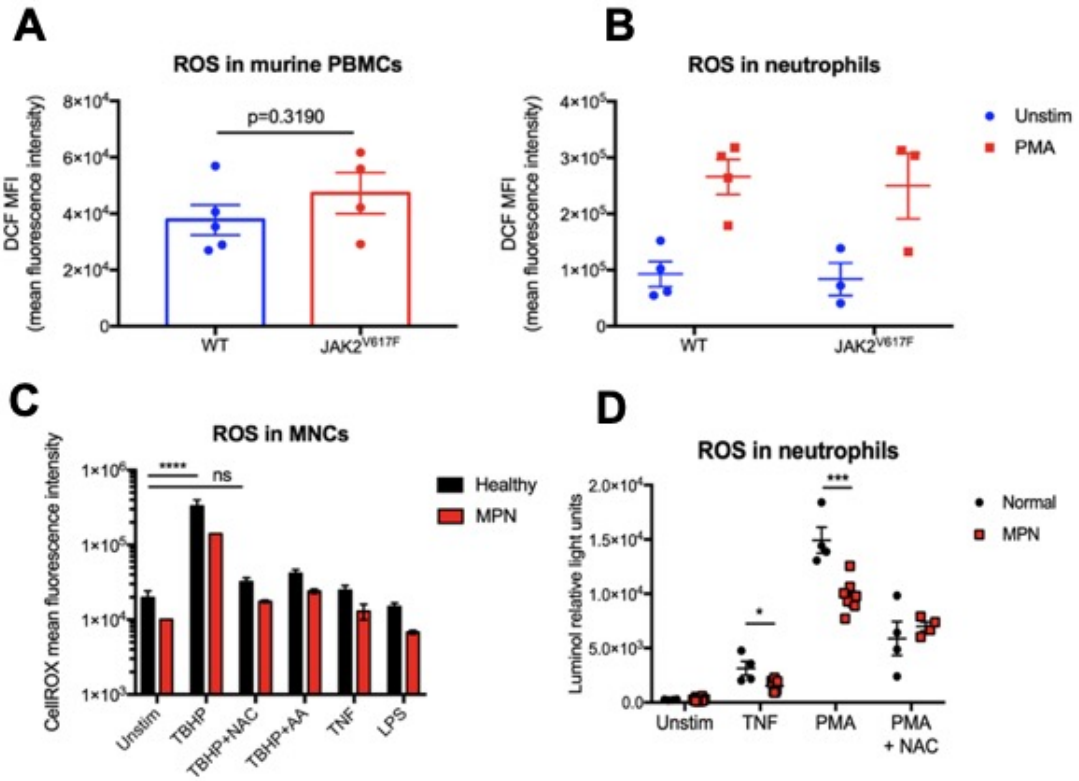

Figure S3.5. Reactive oxygen species in peripheral blood mononuclear cells (PBMCs or MNCs) or primary neutrophils from in MPN mice and humans. (A) Dichlorodihydrofluorescein (DCF) mean fluorescence intensity (MFI) in unstimulated murine PBMCs. N=4-5/group, p=0.3190, t-test. (B) DCF MFI of wild-type and JAK2V617F neutrophils at baseline and following PMA stimulation. (C) CellROX MFI in MPN versus healthy MNCs following stimulation with tert- butyl hydroperoxide (TBHP) +/– N-acetylcysteine (NAC), arachidonic acid (AA), TNF or LPS (50ng/mL each), n=2-4/group, ****p<0.0001 2way ANOVA (D) Luminol-based assay to detect ROS in MPN versus healthy neutrophils following stimulation with TNF or PMA +/– NAC, n=4-7/group, *p<0.05, ***p<0.001 2way ANOVA. Representative data from five biological replicates.

CHAPTER 4

While NAC may prove beneficial to treat common complaints and complications of MPN, it does not selectively target the JAK2 mutant cells. In the next chapter, we make use of common mechanisms of apoptosis evasion in cancer cells to target and eliminate JAK2 mutant cells. Cancer cells can evade programmed cell death by upregulating inhibitor of apoptosis proteins (IAPs) which can then bind to caspases and prevent apoptosis. IAPs can be inhibited through intrinsic pathways following DNA damage by chemotherapy or irradiation where second mitochondria-derived activator of caspases (SMAC) are released into the cytosol and bind to IAPs, leading to apoptosis. Researchers have capitalized on this mechanism by developing small molecule SMAC mimetics, which sensitizes cells that have elevated IAP expression to apoptosis. The SMAC mimetic LCL-161 inhibits multiple IAPs and has been investigated for the treatment of other hematologic malignancies including multiple myeloma and acute myeloid leukemia.

Here, we performed in vitro biochemical assays to measure apoptosis and survival in murine and human JAK2 mutant cell lines as well as calreticulin (CALR) mutant cells. We performed colony forming assays to compare drug's effect on murine and human myeloid progenitor survival. Finally, we treated a transduction transplantation mouse model of MPN with LCL-161 to determine effects in vivo. LCL-161 reduced splenomegaly and modestly reduced mutant cells in the bone marrow. JAK2 activation was necessary for sensitivity to the SMAC mimetic and presence of TNFα eliminated this sensitivity. Interestingly, CALR mutant cells did not exhibit sensitivity to the SMAC mimetic. Thus, our data suggests that driving apoptosis in JAK2 mutant cells with SMAC mimetics could be one therapeutic approach for the treatment of MPN and warrants further investigation.

Article

The SMAC mimetic LCL-161 selectively targets JAK2^{V617F} mutant cells

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Abstract

Background: Evasion from programmed cell death is a hallmark of cancer and can be achieved in cancer cells by overexpression of inhibitor of apoptosis proteins (IAPs). Second mitochondria-derived activator of caspases (SMAC) directly bind to IAPs and promote apoptosis; thus, SMAC mimetics have been investigated in a variety of cancer types, particularly in diseases with high inflammation and NFκB activation. Given that elevated TNFα levels and NFκB activation is a characteristic feature of myeloproliferative neoplasms (MPN), we investigated the effect of the SMAC mimetic LCL-161 on MPN cell survival in vitro and disease development in vivo.

Methods: To investigate the effect of the SMAC mimetic LCL-161 in vitro, we utilized murine and human cell lines to perform cell viability assays as well as primary bone marrow from mice or humans with JAK2–driven MPN to interrogate myeloid colony formation. To elucidate the effect of the SMAC mimetic LCL-161 in vivo, we treated a JAK2^{V617F}–driven mouse model of MPN with LCL-161 then assessed blood counts, splenomegaly, and myelofibrosis.

Results: We found that JAK2^{V617F}-mutated cells are hypersensitive to the SMAC mimetic LCL-161 in the absence of exogenous TNFα. JAK2 kinase activity and NFκB activation is required for JAK2^{V617F}-mediated sensitivity to LCL-161, as JAK or NFκB inhibitors diminished the differential sensitivity of JAK2^{V617F} mutant cells to IAP inhibition. Finally, LCL-161 reduces splenomegaly and may reduce fibrosis in a mouse model of JAK2^{V617F}-driven MPN.

Conclusion: LCL-161 may be therapeutically useful in MPN, in particular when exogenous TNFα signaling is blocked. NFκB activation is a characteristic feature of $JAK2^{V617F}$ mutant cells and this sensitizes them to SMAC mimetic induced killing even in the absence of TNFα. However, when exogenous TNFα is added, NFκB is activated in both mutant and wild-type cells, abolishing the differential sensitivity. Moreover, JAK kinase activity is required for the differential sensitivity of $JAK2^{V617F}$ mutant cells, suggesting that the addition of JAK2 inhibitors to SMAC mimetics would detract from the ability of SMAC mimetics to selectively target $JAK2^{V617F}$ mutant cells. Instead, combination therapy with other agents that reduce inflammatory cytokines but preserve JAK2 signaling in mutant cells may be a more beneficial combination therapy in MPN.

Keywords: Myeloproliferative neoplasm, SMAC mimetic, TNFα

Background

Evasion of programmed cell death is a common survival technique utilized by cancer cells and one way these cells can evade apoptosis is by upregulating anti-apoptotic proteins such as inhibitor of apoptosis proteins (IAPs) [1]. IAPs inhibit apoptosis by directly binding to and inhibiting pro-apoptotic caspases. However, when a cell is triggered to undergo apoptosis, second mitochondria-derived activator of caspases (SMAC) is released into the cytosol and bind IAP proteins, leading to their degradation. Degradation of IAP proteins induces apoptosis by freeing pro-apoptotic caspases and allowing them to induce cell death. Small molecules which mimic the antagonistic effects of SMAC and lead to degradation of IAP proteins, termed SMAC mimetics, are actively being investigated in both hematologic malignancies and solid tumors [2, 3]. In preclinical studies, SMAC mimetics induce apoptosis in cancer cells directly or indirectly by priming these cells for killing by other cytotoxic agents. SMAC mimetics sensitize cells to TNFα-

induced cell death [4]. TNFα is a master regulator of inflammation and induces NFκB activation among other signaling pathways. However, NFκB activation is critical for SMAC mimetic-induced apoptosis [5]. In a study involving primary AML samples, sensitivity to a SMAC mimetic correlated with activation of TNF signaling and low XIAP expression [6]. Therefore, SMAC mimetics may be particularly useful in cancers associated with high inflammation and NFκB activation.

Myeloproliferative neoplasm (MPN) is a chronic hematologic malignancy characterized by chronic inflammation [7–9], high TNFα levels in serum [10], activation of NFκB [11] and reduced expression of XIAP [12]. MPN is characterized by the somatic acquisition of a mutation in either *JAK2* ($JAK2^{V617F}$) [13–17] or calreticulin (*CALR*) [18, 19] in a hematopoietic stem cell. This mutant clone expands, producing excessive numbers of mature myeloid cells that also carry the driver mutation. The current goals of therapy in MPN are reduction of thrombotic risk, reduction in spleen volume, and alleviation of symptoms. There are no pharmacologic therapies currently in use apart from interferon-alpha that specifically deplete the neoplastic clone and lead to molecular remission [20]. Thus, there is an unmet need to identify therapeutics to successfully target $JAK2^{V617F}$ cells in MPN. The SMAC mimetic LCL-161 is currently in a Phase 2 clinical trials for the MPN subtype myelofibrosis (NCT02098161) [21]. However, it still remains unclear how LCL-161 impacts $JAK2^{V617F}$ or *CALR* mutant cells compared to wildtype counterparts.

Based upon the fact that there is hyperactivation of NFκB signaling pathway [22] we reasoned that MPN cells would be more susceptible to killing by SMAC mimetics. To test this, we performed cell viability assays using murine and human cell lines, myeloid colony formation assays using primary MPN patient cells and a mouse model to test the effect of the SMAC mimetic

LCL- 161 on JAK2^{V617F} cells in the presence and absence of TNFα. Here, we find that murine and human JAK2^{V617F} cell lines are hypersensitive to the SMAC mimetic LCL-161 in vitro in the absence but not presence of TNFα. Furthermore, LCL-161 reduced myeloid colony formation to a greater extent in MPN mice and patients and reduced spleen size and possibly myelofibrosis in vivo.

Methods

Drug formulation

LCL-161 was provided by Novartis Pharmaceutical Corporation. For in vitro assays, LCL-161 was dissolved in DMSO at a concentration of 10 mM, stored in single use aliquots at −80 °C, and dissolved directly in culture media fresh for each experiment. LCL-161 was formulated for oral gavage by dissolving in 0.1 N HCl to a final concentration of 10 mg/ml in sodium acetate buffer (100 mM, pH 4.6). LCL-161 was prepared by first wet- ting 10 mg powder stock with 30 µl water, and then dis- solving in two equivalents (0.73 µl/mg of compound) of 6.0 N HCl. The resulting (clear) solution was brought up to 1 ml in pH 4.6 acetate buffer, and the resulting stock was stored frozen at −20 °C until used for gavage treatment of mice.

Creation of L929 cell lines

Parental L929 cell line (ATCC CCL-1, purchased from ATCC) were infected with retrovirus containing JAK2^{WT}, JAK2^{V617F}, CALR^{DEL}, CALR^{WT}, MPL, or empty vector containing green fluorescent protein (GFP) or human CD4 (hCD4) tags by spinoculation. GFP+ and/or hCD4+ cells were sorted on an Aria flow cytometer (BD) and expanded in culture.

Protein extraction and Western Blot

Following washes with PBS, cells were lysed in 1× Immunoprecipitation buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 10 mM α-glycerophosphate, 1 mM Na_3VO_4, and 50 mM NaF) by vortexing for 10 s and placing cells on ice for 10 min. Next, samples were centrifuged at 12,000×*g* for 15 min at 4° C. Supernatants were collected and 4× Lamelli buffer (Bio-Rad) was added to each sample prior to stor- age at −20 °C. Protein concentration was determined by Bicinchoninic acid (BCA) assay at absorbance at 532 nm. Western blot analysis was performed using 30 μg for protein boiled for 10 min at 95 °C and electrophoresis was carried out on a 10% acrylamide gel. The protein was transferred onto a nitrocellulose membrane and blocked with 5% BSA in 0.1% PBS-T at 25 °C. The membrane was incubated for 1 h at room temperature or overnight at 4 °C with the primary antibodies for cIAP1 (ab2399, Abcam), cIAP2 (ab23423, Abcam), XIAP (ab28151, Abcam), and β-actin (ab8227, Abcam). Following three 15 min wash steps with 0.1% PBS-T, membranes were incubated with secondary antibody goat-anti-rabbit IgG for 1 h. Following an additional wash step, mem- branes were imaged on a Chemdoc luminescence reader (Bio-Rad).

Human cell lines and apoptosis assays

K562 and HEL cells (gift from Brian Druker Lab, Oregon Health & Science University) were maintained in RPMI 1640 (Corning) containing phenol red supplemented 100 U penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FBS. Cells were cultured in a humidified tissue culture incubator at 37 °C and 5% CO_2. For apoptosis assays, K562 and HEL cells were plated into 12-well plates at a density of 1 × 10 cells per well in RPMI + 10% FBS + Pen/Strep/l-glutamine containing vehicle, LCL-161, or LCL-161 + TNFα. Following 16h, flow cytometry for annexin-V and propidium iodide (PI) was performed.

Murine colony formation assays

Bone marrow was harvested from JAK2^{V617F} knock-in mouse model of MPN and cells were plated at a density of 10,000 cells/ml in Methocult M3230 (stem cell) supplemented 10 ng/ml mouse interleukin-3 (mIL-3), 3 U/ml human erythropoietin (hEPO), and 50 ng/ml mouse stem cells factor (mSCF), all purchased from Peprotech. The cells were plated in triplicate at a density of 10,000 cells/ ml and incubated at 37 °C in a 5% carbon dioxide humidified incubator for 5–7 days. Standard morphological criteria were used to score hematopoietic colonies using a light microscope.

Collection, isolation and culture of primary human samples

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 Board of the University of California, Irvine (IRB HS #2014-9995). Peripheral blood samples were collected from MPN patients with Polycythemia Vera, Essential Thrombocytopenia, and Primary Myelofibrosis or normal controls. Density gradient centrifugation with Ficoll was performed to isolate blood mononuclear cells (PBMCs). Following PBMC isolation, a red blood cell lysis step was performed with ammonium chloride buffer (ACK).

Human hematopoietic colony assays

Isolated PBMCs were plated in Methocult H4320 (Stem Cell Technologies, Vancouver Canada) supplemented 10 ng/ml human interleukin-3 (hIL-3), 3 U/ml human erythropoietin (hEPO), and 50 ng/ml human stem cells factor (hSCF). The cells were plated in triplicate at a density of 100,000 cells/ml and incubated at 37 °C in a 5% CO_2 humidified incubator for 7–10

days. Standard morphological criteria were used to score hematopoietic colonies using a light microscope.

Mice

All mouse work was performed with approval from the IACUC committee at University of California, Irvine. Equal numbers of male and female mice were used for all experiments. The $JAK2^{V617F}$ was a gift from Ann Mullally, Brigham and Women's Hospital. The transduction–transplantation mouse model of $JAK2^{V617F}$ driven-MPN was established as previously described [10, 23].

Statistics

All statistical analyses were performed using Graphpad Prism version 7.0 (Graphpad, La Jolla, CA, USA). Statistical analyses were performed using an unpaired t-test (Holm–Sidak) or 2way ANOVA (Tukey's test). Significant differences were indicated as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Error bars represent mean values ± SEM. All experiments were performed at least three independent times unless otherwise stated.

Results

$JAK2^{V617F}$ mutant cells are hyper-sensitive to the SMAC mimetic LCL-161 in the absence but not presence of TNFα

To investigate the sensitivity of $JAK2^{V617F}$ mutant cells to LCL-161, we created cell lines with stable expression of $JAK2^{V617F}$, $JAK2^{WT}$, or empty GFP vector by retroviral transduction. In this assay, we utilized the mouse fibroblast L929 line which are extremely sensitive to TNFα-

induced death. We measured the impact of increasing concentrations of LCL-161 on the viability of these cells using a resazurin based assay in the presence or absence of TNFα (Fig. 4.1a–c). Sub-micromolar concentrations of LCL-161 significantly reduced the viability of L929 JAK2^{V617F} cells compared to JAK2^{WT} or empty vector cells (Fig. 4.1a). We observed 50% cell viability of L929 JAK2^{V617F} cells with 0.45 μM LCL-161 as compared to similar cell viabilities with 5.5 μM and 9.5 μM LCL-161 for empty vector or JAK2^{WT} cells, respectively (P<0.0001, 2way ANOVA; Fig. 4.1a). However, this differential sensitivity was lost when TNFα was added to culture (Fig. 4.1b). Interestingly, overexpression of JAK2^{WT} rendered L929 cells resistant to killing by higher concentrations of LCL-161 in the presence of TNFα, as these cells were 50% viable in the presence of 19 μM LCL-161 (P < 0.0001, 2way ANOVA; Fig. 4.1b).

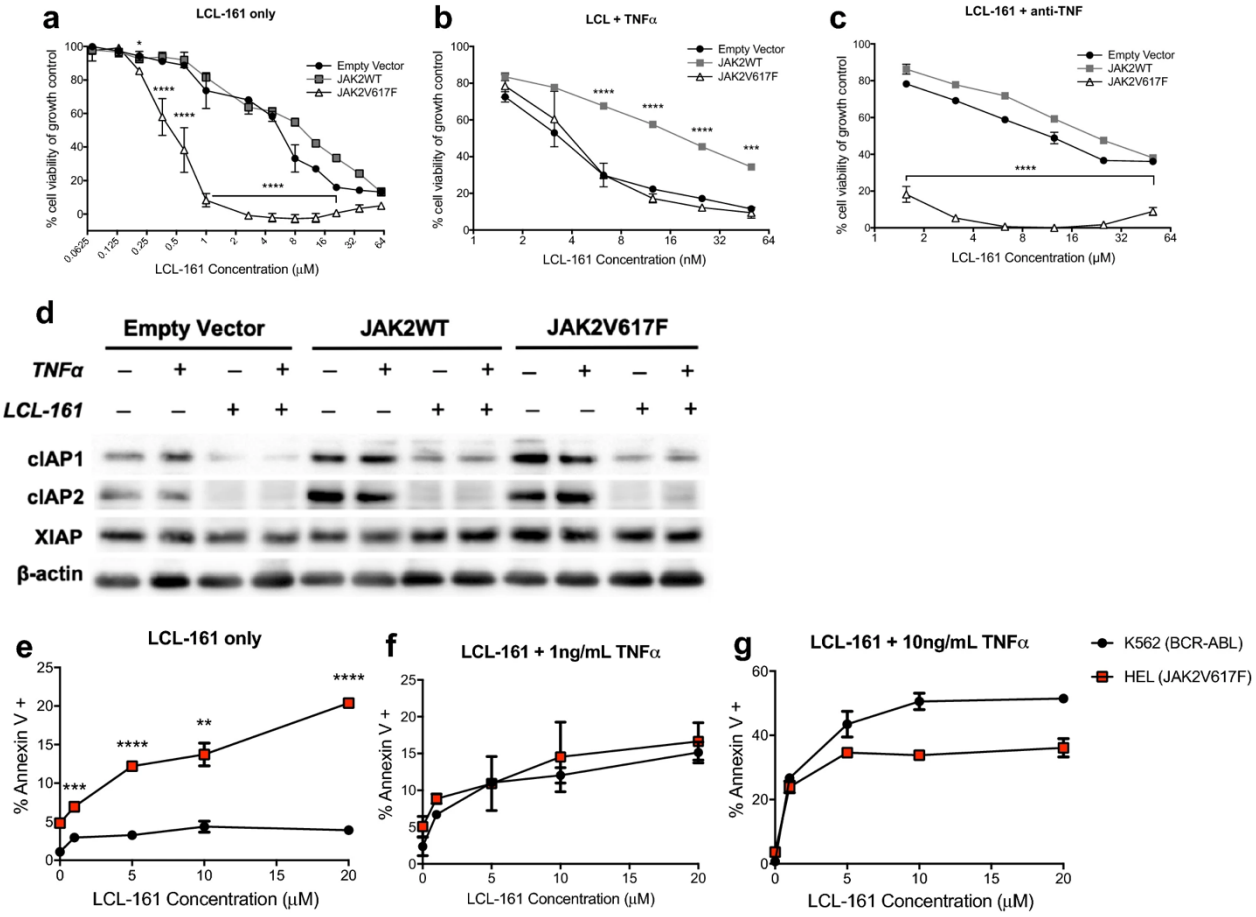

Figure. 4.1. JAK2^{V617F} mutant cells lines are more sensitive to killing by LCL-161 under certain circumstances. a–c. L929 cells expressing JAK2^{WT}, JAK2^{V617F} or empty vector were incubated with increasing concentrations of **a.** LCL-161 alone, **b**. LCL-161 with the addition of 0.25ng/ml mTNFα, **c**. LCL-161 with the addition of 400ng/ml mTNFa neutralizing antibody for 48 h and then analyzed with a resazurin based viability assay. ****$P < 0.0001$, 2way ANOVA. **d** Western blot of L929 cell lines harvested 24 h after exposure to combinations of LCL-161 and mTNFα. **e**–**g**. HEL and K562 cells were incubated with **e**. LCL-161 alone, **f**. LCL-161 + 1ng/ml hTNFα, or **g**. LCL-161 + 10ng/ml hTNFα for 48 h. Apoptosis was measured with Annexin V and PI staining. **$P < 0.01$, ****$P < 0.0001$ unpaired t test.

Next, we assessed whether autonomous production of TNFα could explain the increased sensitivity of JAK2^{V617F} cells to LCL-161. To do this, we tested whether TNFα neutralizing antibody abrogates the increased sensitivity of JAK2^{V617F} mutant L929 cells to LCL-161. Addition of TNFα neutralizing antibody did not rescue the increased sensitivity of JAK2^{V617F} cells to LCL-161 (Fig. 4.1c) suggesting that the sensitivity of JAK2^{V617F} cells was not due to autonomous production of TNFα. Moreover, we could not detect any TNFα in the supernatant of L929 JAK2^{V617F} cells, either at baseline or with the addition of LCL-161 by ELISA (data not shown). As expected, treatment with LCL-161 decreased both cIAP1 and cIAP2 (Fig. 4.1d). We found no marked differences in expression of cIAP1, cIAP2, or XIAP in JAK2^{V617F} versus JAK2^{WT} or parental L929 cells under any conditions tested (Fig. 4.1d). However, we did find evidence that sensitivity to LCL-161 is mediated at least in part to NFκB activation, the NFκB inhibitor QNZ had a protective effect on empty vector cells in the presence but not absence of TNFα, whereas QNZ had a protective effect on JAK2^{V617F} mutant cells only in the absence of TNFα (Figure S4.1).

This suggests that JAK2^{V617F} induces NFκB activation in L929 cells and this mediates the sensitivity to LCL-161. We also compared the ability of LCL-161 to induce apoptosis in the HEL human erythroleukemia cell line which harbors the *JAK2^{V617F}* mutation versus the K562 human erythroleukemia cell line which harbors the *BCR-ABL* translocation. LCL-161 induced apoptosis significantly more in HEL cells as compared to K562 cells in the absence (P<0.001, unpaired t test; Fig. 4.1e) but not presence of TNFα (Fig. 4.1f, g). Likewise, expression levels of cIAP1,

cIAP2, or XIAP did not correlate with sensitivity to LCL-161 in HEL human cells expressing JAK2^{V617F} as compared to K562 cells (Figure S4.2). These data in human cell lines corroborate our findings in L929 cells that JAK2^{V617F} mutant cells are more sensitive to killing by LCL-161 alone, but when TNFα is added this differential sensitivity is lost.

Calreticulin mutant cells are not hyper-sensitive to LCL-161

To investigate the effect of LCL-161 on calreticulin mutant cells, we also created L929 cell ectopically expressing MPN associated calreticulin (CALR) mutations with and without its obligate cytokine receptor scaffold thrombopoietin receptor (MPL). CALR mutant L929 cells were not more sensitive to LCL-161 as compared to empty vector either with or without co-expression of MPL (Figure S4.3). Interestingly, overexpression of wild-type CALR led to increased sensitivity to LCL-161 when co-expressed with MPL as compared to cells with empty vector, CALR deletion or CALR insertion (Figure S4.3).

Sensitivity of JAK2^{V617F} cells to LCL-161 is dependent upon Janus kinase activity

To determine whether the increased sensitivity of JAK2^{V617F} cells to LCL-161 is dependent upon Janus kinase (JAK) activity, we utilized the JAK inhibitors ruxolitinib (JAK1/2 inhibitor) and pacritinib (JAK2/Flt3 inhibitor) in a resazurin based cell viability assay. Sensitivity of JAK2^{V617F} L929 cells was dependent upon activation of JAK2 activity, as treatment with either ruxolitinib or pacritinib reversed the increased sensitivity of JAK2^{V617F} cells to LCL-161 (Fig. 4.2). This data suggests that the hyper-sensitivity of JAK2^{V617F} cells to LCL-161 is due to constitutive JAK2 activation.

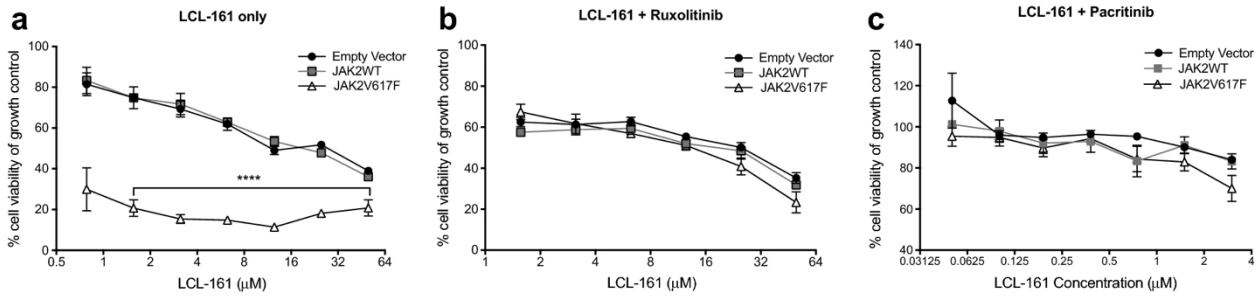

Figure 4.2. JAK inhibitors rescue hypersensitivity of JAK2^{V617F} mutant cells to LCL-161. L929 cells expressing JAK2^{WT}, JAK2^{V617F} or empty vector were incubated with increasing concentrations of **a**. LCL-161 alone, **b**. LCL-161 with the addition of 1μM ruxolitinib, or **c.** LCL-161 with the addition of 1μM pacritinib. After 48h in culture a resazurin-based cell viability assay was performed. ****$P < 0.0001$, 2way ANOVA.

LCL-161 reduces myeloid colony formation in JAK2^{V617F} knock-in mice and MPN patient cells in the absence of TNFα

To investigate the effect of LCL-161 on myeloid colony formation in primary mouse bone marrow cells, we performed colony formation assays from JAK2^{V617F} knock-in and wild-type mice with increasing concentrations of LCL-161. LCL-161 reduced colony formation in JAK2^{V617F} cells compared to wild-type cells in the presence of all doses tested, although only the 1 μM LCL-161 dose reached statistical significance ($P < 0.001$, unpaired t-test; Fig. 4.3a). However, the differential sensitivity of JAK2^{V617F} cells was lost in the presence of TNFα (Fig. 4.3b). This data further supports the model that JAK2^{V617F} cells have increased sensitivity to SMAC mimetics in the absence but not presence of TNFα.

We next investigated the effect of LCL-161 on colony formation using primary cells from MPN patients compared to unaffected individuals. In the presence of LCL-161 alone, there was a reduction in colony formation from JAK2^{V617F} mutated MPN patients versus normal controls at 1 μM and 2.5 μM doses, though this trend did not reach significance ($P = 0.05$ and 0.07, respectively, unpaired t test; Fig. 4.3c). These same trends were observed when erythroid and G/M colonies

were enumerated separately (Figure S4.4). When TNFα was added to the culture, this diminished the apparent difference between MPN and normal controls in response to LCL-161 at all but the highest concentration (5 μM) of LCL-161 ($P < 0.05$, unpaired t test; Fig. 4.3d).

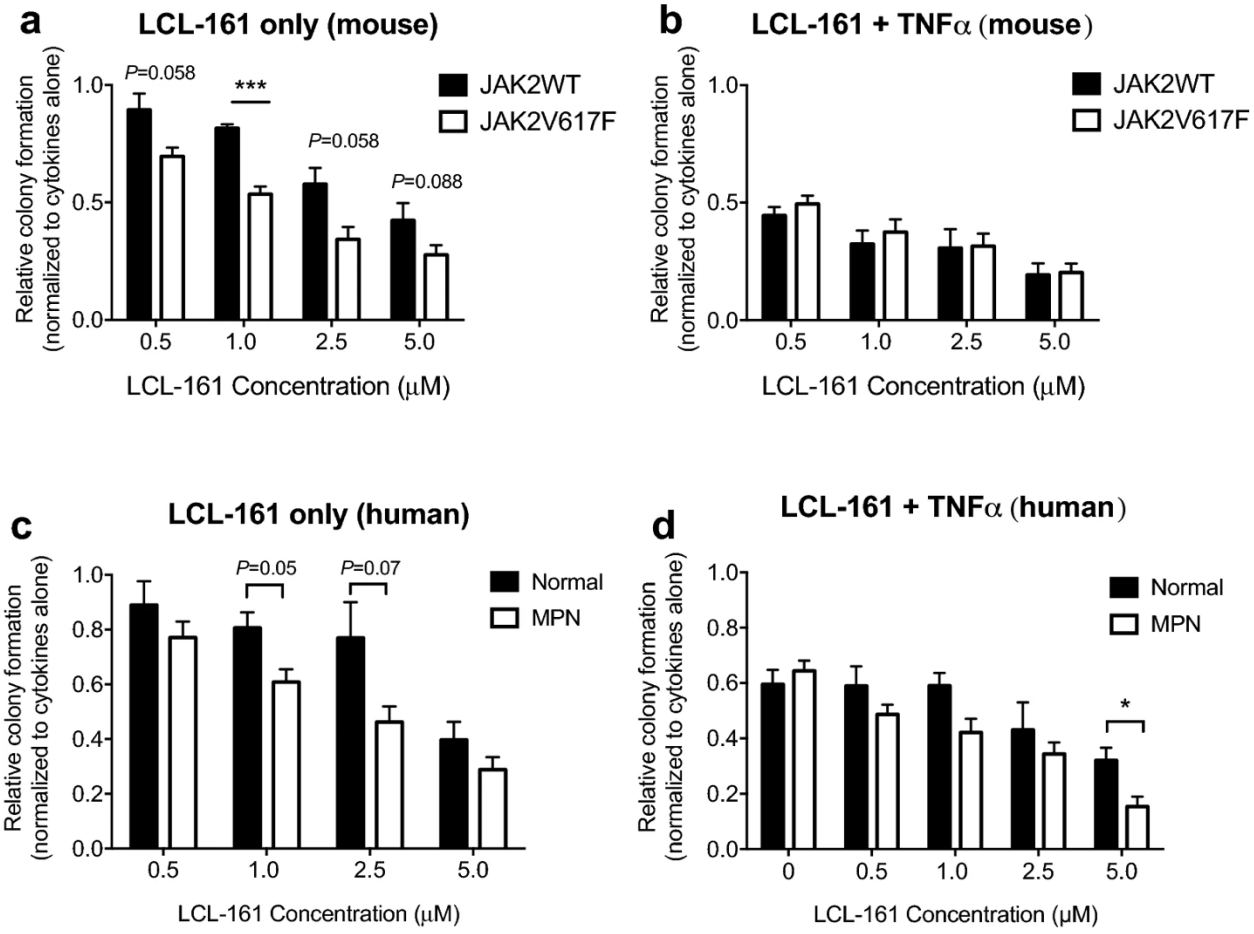

Figure 4.3. LCL-161 preferentially decreases MPN colony formation. Methylcellulose colony formation of **a**, **b.** whole bone marrow from JAK2^{V617F} or wild-type mice and **c**, **d.** peripheral blood mononuclear cells from *JAK2^{V617F}* mutated MPN patients or normal controls with increasing concentrations of LCL-161 alone (**a**, **c**) or LCL-161 + 0.25 ng/ml TNFα (**b**, **d**). *$P < 0.05$, ***$P < 0.001$, unpaired t test.

LCL-161 reduces spleen size and ameliorates fibrosis in a JAK2^{V617F} transduction–transplantation model

To test the efficacy of LCL-161 in vivo, we utilized a JAK2^{V617F} transduction–transplantation MPN mouse model that develops myelofibrosis around 1 year of age [23,24,25,26].

Treatment with LCL-161 was initiated 15 weeks after transplant with twice weekly oral administration. We did not detect changes in peripheral blood counts nor percentage of GFP+ cells in the peripheral blood in LCL-treated mice compared to untreated mice (n = 3–4 mice/group, Fig.4.4a–e). However, at sacrifice we found that LCL-161 treatment significantly reduced spleen size ($P < 0.05$, unpaired t test; Fig.4.4f) and modestly reduced the percentage of GFP+ cells in the bone marrow and spleen of JAK2^{V617F} mice (n = 3–4 mice/group, Fig.4.4g). Upon analysis of reticulin-stained bone marrows, we observed that fibrosis was reduced in mice that had been treated with LCL-161 (Fig.4.4h). These data demonstrate that LCL-161 may have a beneficial effect on an in vivo model of JAK2^{V617F} mutated MPN.

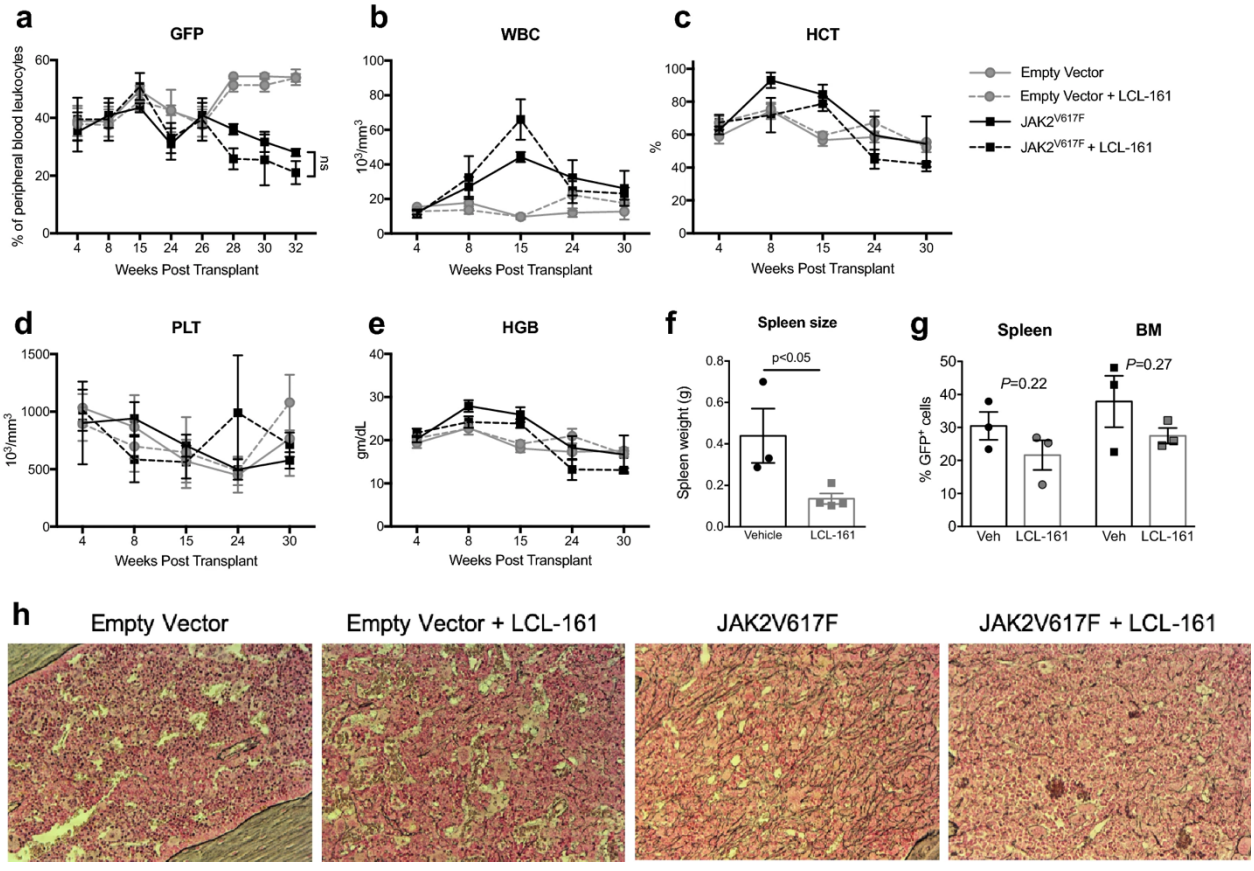

Figure 4.4. Impact of LCL-161 treatment in a transduction–transplantation model of JAK2V617F mutated MPN. a Percentage of GFP+ (empty vector or JAK2^{V617F}) cells in peripheral blood. **b** White blood cell (WBC), **c** hematocrit (HCT), **d** platelet (PLT) and **e** hemoglobin (HGB) counts in wild-type (empty vector) or JAK2^{V617F} mice treated with LCL-161. **f** Spleen weights in JAK2^{V617F} mice, $P < 0.05$, unpaired t test. **g** Percentage of GFP+

(JAK2^{V617F}) cells in the spleen and bone marrow. **h** Snook's reticulin staining of paraffin-embedded bone marrows in wildtype (empty vector) and JAK2^{V617F} mice treated with LCL-161. n = 3–4 mice per group.

Discussion

Here, we found that mouse cell lines ectopically expressing JAK2^{V617F}, the *JAK2^{V617F}*-mutated human cell line HEL, JAK2^{V617F} knock-in mice, and primary MPN samples were more sensitive to killing by the SMAC mimetic LCL-161 compared to their JAK2^{WT} counterparts. Although addition of exogenous TNFα increased the sensitivity of both mutant and wild-type cells to LCL-161, the differential sensitivity of mutant cells was abolished when TNFα was added to culture. The increased sensitivity to LCL-161 was not due to autocrine production of TNFα, as pharmacologic blockade of TNFα did not rescue JAK2^{V617F} mutant cells from LCL-161.

Activation of JAK2 signaling is critical for the increased sensitivity of JAK2^{V617F} mutant cells to LCL-161. Addition of JAK2 inhibitors ruxolitinib or pacritinib restored LCL-161 sensitivity of JAK2^{V617F} mutant to that of wild-type cells. This observation suggests JAK2 inhibitors may reduce the vulnerability of JAK2 mutant cells to be preferentially killed by SMAC mimetics and that therapeutic combinations of JAK2 inhibitors and SMAC mimetic in patients may not be an ideal combination. This is an important point given that it is common for new investigational agents in myelofibrosis to be tested in combination with ruxolitinib.

The ex vivo experiments with mouse and primary human myeloid colony formation corroborate our findings in cell lines that MPN mutant cells are more sensitive than wild-type cells in the absence but not presence of TNFα. Human MPN patients have variable frequencies of JAK2^{V617F} and so our methylcellulose colony formation assays with primary MPN patient cells assess both mutant and wild-type colonies. This may explain why the impact of LCL-161 on MPN

patient colony formation was less robust than our observations in cell lines bearing the $JAK2^{V617F}$ mutation.

Cells bearing the MPN associated $CALR^{DEL}$ mutation were not more sensitive to LCL-161 when expressed either with or without its obligate cytokine receptor scaffold MPL. This suggests that SMAC mimetics may be most beneficial in $JAK2^{V617F}$ mutated MPN patients. CALR binds to and specifically activates MPL, whereas $JAK2^{V617F}$ activates signaling of multiple cytokine receptor pathways. The sensitivity of $JAK2^{V617F}$ to SMAC mimetics is likely mediated via activation of pathways distinct from MPL. Therefore, combination therapy with SMAC mimetic and drugs which target MPL signaling may be additive or even synergistic in $JAK2^{V617F}$ mutated MPN.

In our in vivo mouse model, we found that LCL-161 had modest effects on peripheral blood counts. Although LCL-161 did reduce the percentage of GFP+ cells in the spleen and bone marrow, these effects were modest and did not reach significance. The primary benefit of LCL-161 in this mouse model was reduction in splenomegaly and possible reduction of fibrosis. Future studies would require increasing numbers of mice to validate the effect of LCL-161 on splenomegaly and myelofibrosis in vivo. Regardless, our findings suggest that LCL-161 may not be an ideal single agent in MPN but may be most useful as an adjunct to other MPN therapies.

Approaches that reduce TNFα but maintain constitutive activation of JAK2 in mutant cells may be an ideal combination with SMAC mimetics in MPN. The TNFα decoy receptor etanercept has been investigated as a single agent in an open label pilot study in myelofibrosis and was found to alleviate symptoms [27]. Another potential approach as a combinatorial study with SMAC mimetics would be to combine with a JAK inhibitor that is devoid of JAK2 inhibitory activity,

such as itacitinib (INCB039110). This would result in reduction of inflammatory cytokines including TNFα while preserving the vulnerability of $JAK2^{V617F}$ cells to killing by SMAC mimetic.

Conclusion

SMAC mimetics may be useful in patients with $JAK2^{V617F}$ mutated MPN, as $JAK2^{V617F}$ -mutant cells are more readily killed by SMAC mimetics as compared to wild-type cells. This vulnerability to SMAC mimetics is mediated via JAK2 activation, therefore addition of a JAK2 inhibitor to SMAC mimetics may detract from the ability of SMAC mimetics to preferentially kill $JAK2^{V617F}$ mutant cells. However, therapy with agents that reduce or block TNFα may augment the ability of SMAC mimetics to specifically target $JAK2^{V617F}$ mutant cells.

Abbreviations: BCA: bicinchoninic acid assay; BSA: bovine serum albumin; CALR: calreticulin; EPO: erythropoietin; FBS: fetal bovine serum; GFP: green fluorescent protein; G/M: granulocyte/monocyte; IAP: inhibitor of apoptosis proteins; IL-3: interleukin-3; JAK: Janus kinase; MPL: thrombopoietin receptor; MPN: myeloproliferative neoplasm; NFKB: nuclear factor kappa-light-chain-enhancer of activated B cells; PI: propidium iodide; PBMNC: peripheral blood mononuclear cells; SCF: stem cell factor; SMAC: second mitochondria-derived activator of caspases; TNFα: tumor necrosis factor-alpha; PBS: phosphate buffered saline.

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Authors' contributions: BMC analyzed the data, designed the figures and wrote the manuscript. TKN, JN, CH and HN performed experiments and analyzed the data. SJM discussed results and provided comments and feedback. AGF planned, designed, per- formed experiments, and coordinated the research and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: All primary human samples used in this study were derived from participants who consented to an IRB approved study "Role of Inflammation in Myeloproliferative Neoplasm" at University of California, Irvine. All animal use in this protocol was in accordance with an approved IACUC protocol.

Consent for publication: All authors have reviewed the manuscript and consent for publication.

Competing interests: The authors declare that they have no competing interests.

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Supplemental

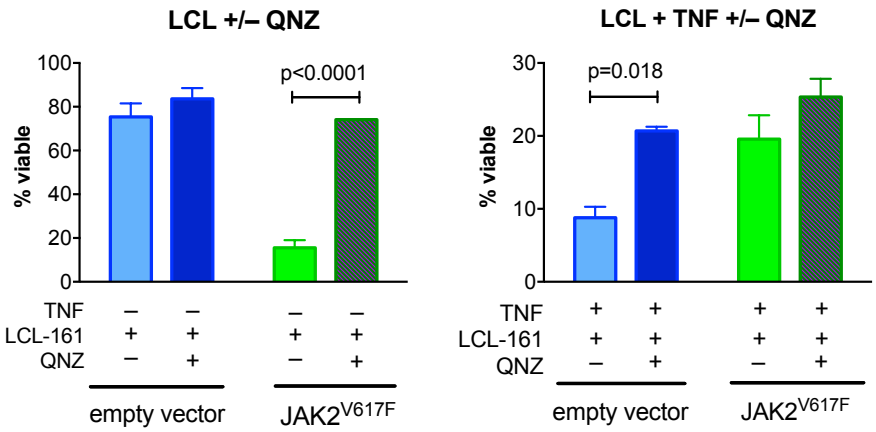

Figure S4.1. NFκB inhibitor protects JAK2^{V617F} mutant cells from killing by LCL-161 in the absence of TNFα and empty vector cells from killing by LCL-161 in the presence of TNFα. L929 cell transduced with empty vector or JAK2^{V617F} were exposed to LCL-161, either in the presence or absence of 1 ng/ml hTNFα and 1nM QNZ. The concentration of LCL-161 used without TNFα was 1.5 μM and with TNFα was 12.5 nM. After 48 h of culture a resazurin based cell viability assay was performed.

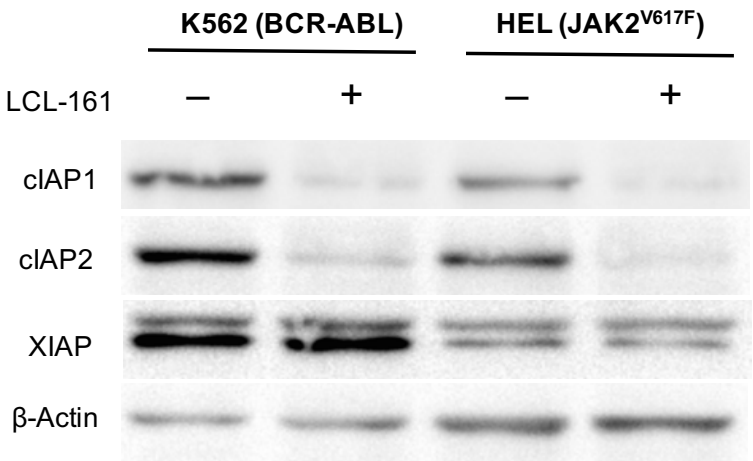

Figure S4.2. Expression levels of cIAP1/2 or XIAP do not influence sensitivity of human cell lines to LCL-161. Western blot of K562 (BCR-ABL mutant) and HEL (JAK2^{V617F}) cells treated with 10 nM LCL-161 for 2 h before protein isolation. LCL-161 reduces expression of cIAP1 and cIAP2 in both cell lines as expected. XIAP expression was unaffected by LCL-161.

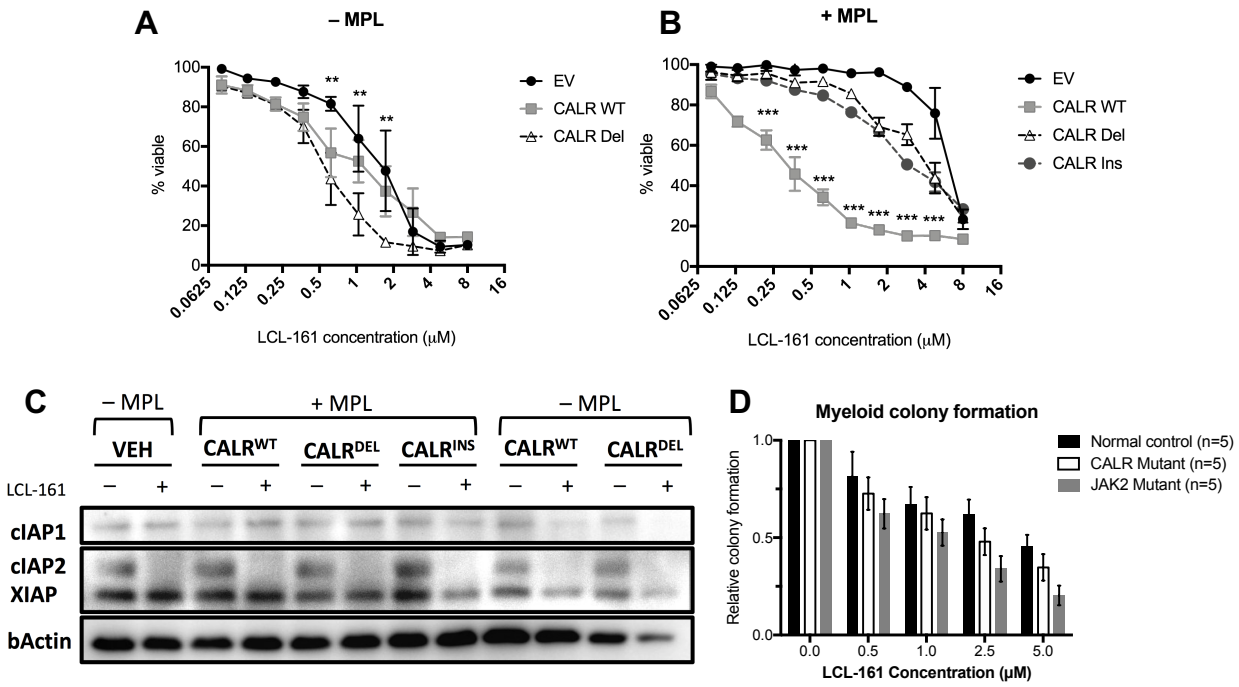

Figure S4.3. Calreticulin-mutant cells are not hyper-sensitive to LCL-161. (A, B) Resazurin-based cell viability assay showing L929 cells transduced with the Calreticulin (CALR) mutations represent- ing empty vector (EV), wild-type CALR (CALR^{WT}), deletion (CALR^{DEL}) or insertion (CALR^{INS}) (A) containing thrombopoietin receptor (MPL) and (B) without MPL treated with increasing concentrations of LCL-161 for 48 h. **P < 0.01, ***P < 0.001 2way ANOVA. (C) Western blot for cIAP1/2, XIAP, and β-Actin as a loading control in CALR^{WT}, CALR^{DEL}, CALR^{INS}, or empty vector (VEH) cells in the presence or absence of MPL. (D) Myeloid colony formation using MNCs from normal controls (n = 5), CALR-mutated patients (n = 5), and JAK2^{V617F} patients (n = 5). Cells were plated in methylcellulose with varying LCL-161 concentrations. Colonies were counted from each plate and normalized to 0 μM LCL-161. Error bar represent mean values ± SEM.

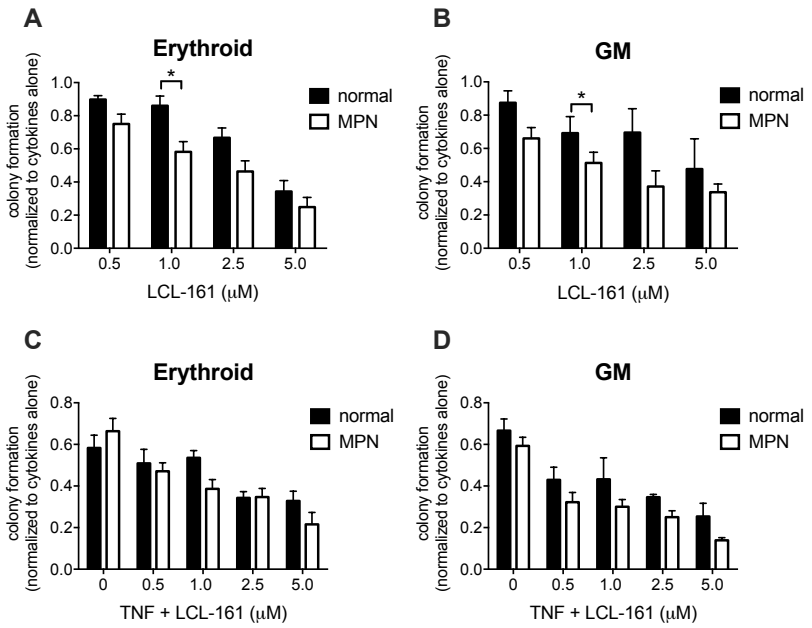

Figure S4.4. Colony formation shown in Fig. 4.3 separated by erythroid and G/M colonies. (A) Erythroid and (B) G/M colony formation from MPN patients and normal controls with increasing concentrations of LCL-161. (C) Erythroid and (D) G/M colony formation from MPN patients and normal controls with 10 ng/ml TNFα + increasing concentrations of LCL-161.

CHAPTER 5

Summary and Conclusions

In this dissertation, we investigated the impact of inflammation on multiple immune cell types during many functions including hematopoietic stem and progenitor cell proliferation and fitness during emergency myelopoiesis, platelets and leukocytes during thrombosis generation and erythroleukemia cells during apoptosis. Given the importance of JAK-STAT signaling in many immune functions, it's not surprising that aberrant JAK2 signaling has varied effects on cellular functions in vitro and in vivo. The ultimate goal of this project was to identify how JAK2 mutant hematopoietic stem cells respond to and function compared to normal stem cells in an inflammatory setting. Much of this work focused on stem cell responses to lipopolysaccharide, which has been well characterized in the field to drive HSC proliferation and impair cell fitness. Upon LPS-induced inflammation, stem cells exit quiescence to enter cell cycling, correlated with increased oxidative stress. We found that $JAK2^{V617F}$ HSCs are hyperproliferative at baseline compared to the normally quiescent wildtype HSCs, which may be more readily available to respond to inflammation. However, mutant and normal cells exhibited similar rates of proliferation following acute LPS stimulation and this observation could justify why we did not observe a selective advantage of $JAK2^{V617F}$ cells to LPS-induced inflammation.

The current theory in the field suggests that specific HSC mutants will respond differently to various inflammatory stimuli and thus will have a competitive advantage under certain circumstances. For instance, clones with the PPM1D mutation in patients with clonal hematopoiesis have been found to be resistant to apoptosis induced by platinum-based chemotherapy agents but not to other agents that cause DNA damage (18). LPS is a general

pathogen associated molecular pattern that induces the production of multiple pro-inflammatory cytokines. JAK2^{V617F} cells may differentially respond to some but not all inflammatory cytokines that are upregulated by LPS and one or more of these cytokines could be contributing impaired fitness after chronic LPS stimulation in mutant cells. In an attempt to compare the effects of single cytokines on HSC proliferation and/or survival in vitro, we sorted single wildtype or JAK2 mutant HSCs per well in a round bottom Terasaki plate preloaded with media containing an inflammatory cytokine then tracked cell numbers over time (data not shown). Not surprisingly, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 each significantly increased wildtype and JAK2^{V617F} HSC proliferation, though IFNɣ or IL-11 alone increased proliferation of JAK2^{V617F} and not normal HSCs. Unfortunately, these cells lose stemness in culture over time and transplantation of these cells into irradiated hosts was unsuccessful.

Stem cells are defined by the cell's ability to differentiate into multiple cell types as well as to self-renewal in order to maintain for the lifespan of the host. One limitation of this project is that we did not measure whether HSC proliferation was towards self-renewal versus differentiation following LPS stimulation. A future study could include these assays to determine what decisions JAK2 mutant versus normal HSCs make in various settings. Cell cycling is necessary to replenish blood cells following a hematopoietic challenge, yet differentiation comes at the expense of self-renewal. In fact, the most common mutations that occur in patients with clonal hematopoiesis involve epigenetic regulators such as *Tet2* or *DNMT3A* that, when mutated, drive aberrant stem cell self-renewal and lead to increased frequencies over time. A recent mathematical modelling study of *Tet2* mutant HSCs found that chronic inflammation from an atherosclerotic mouse model could drive CH by increasing HSC proliferation and accelerating somatic evolution (19). Thus,

chronic inflammation can promote onset and progression of hematological diseases by driving HSC proliferation and increasing the mutational burden in these cells.

Increased levels of reactive oxygen species (ROS) are associated with hematopoietic stem cell cycle entry. ROS are produced during many biological functions including mitochondrial oxidative phosphorylation and play key roles in cell signaling, though high levels of ROS can be damaging. Previous studies have used knock-out mouse models of key genes involved in oxidative stress including the master regulator nuclear factor erythroid factor 2 (Nrf2) (20). Merchant et al found that HSPCs lacking Nrf2 did not have elevated basal ROS but were sensitive to apoptosis following an induced oxidative stress (20). $Nrf2^{-/-}$ HSCs had impaired differentiation and survival following transplantation that was found to be dependent upon the dysregulated cytokine signaling rather than by aberrant oxidative stress (20). While oxidative stress and cell cycle entry appear to be correlated, our time course data in wildtype mice provides evidence that oxidative stress is not necessary for LPS-induced HSC cycling. Furthermore, oral NAC treatment did not reduce basal ROS in the HSC compartment but did reduce basal ROS in whole bone marrow cells and reduced plasma TNFα following LPS. The reduction of TNFα in NAC treated animals could explain for the spared stem cell fitness following chronic LPS in vivo. Ultimately, these data suggest that elevated oxidative stress in HSCs is not directly linked to cellular dysfunction.

Elevated ROS can damage DNA and damaged DNA is a hallmark of aged stem cells that contributes to functional decline. The surrogate marker for DNA damage is phosphorylation of γH2AX, which is one of the first steps of the DNA damage response by ATM and ATR kinases. Initially, we were surprised to observe robust and rapid γH2AX in HSCs via flow cytometry following an acute LPS stimulation. However, upon visualization of foci by immunofluorescence, we observed pan-H2AX staining in HSCs that was indicative of replication stress rather than true

DNA damage from double strand breaks, in accordance with previous reports (3, 21). Further, γH2AX was transient in HSCs and correlated with proliferation based on BrdU incorporation. Takizawa et al used transgenic knockout mice to identify that LPS-induced replication stress is driven by TLR4 and TRIF, rather than by the MyD88 pathway (15). While others have shown that JAK2 mutant cells have persistent DNA damage foci, our work confirms low levels of γH2AX foci in JAK2^{V617F} stem and progenitor cells at baseline. Future work could explore the differential DNA damage responses in JAK2 mutant and normal stem cells in the context of acute versus chronic inflammation to compare impacts on stem cell functions.

Transplantation is performed to measure hematopoietic stem cell function by tracking multicellular engraftment, though transplantation itself induces stress hematopoiesis and can exhaust HSCs after multiple rounds. To observe cell intrinsic effects of the JAK-STAT activation on stem cell biology, we performed competitive transplants by injecting equal ratios of mutant and WT bone marrow into lethally irradiated recipients. In our hands, JAK2^{V617F} cells don't compete as well as wildtype cells and have reduced engraftment in the blood and bone marrow over time. Interestingly, the hyperproliferative phenotype of mutant cells was lost following transplantation, suggesting that the bone marrow stroma may play a role in stem cell proliferation states. An alternative explanation for reduced HSC proliferation following transplantation is that mutant HSCs become less hyperproliferative with age or stress and we observe an accelerated exhaustion phenotype in transplanted mice with mutant cells. Mice transplanted with JAK2^{V617F} cells appear to be hypersensitive to LPS and can die from acute LPS at otherwise sub-lethal doses (data not shown). LPS is directly sensed by the TLR4 on HSCs or by cytokine sensing from the surrounding niche of macrophages, adipocytes and stroma. It's possible that the JAK2 mutant cells influence the wildtype bystander cells to increase the inflammatory response to LPS. Thus, a few questions

remain whether irradiation, transplantation or the presence of $JAK2^{V617F}$ cells sensitizes hosts to LPS-induced sepsis. It would also be interesting to observe how wildtype HSCs respond to inflammation in a $JAK2^{V617F}$ host, which would be technically challenging but not impossible. Nonetheless, the stress of transplantation and engraftment influences HSC response to acute inflammation.

One key question that remains is how $JAK2^{V617F}$ influences human versus murine HSCs. A feature of human MPN biology not represented in the mouse model is a dampened response to IL-10 signaling. Upon stimulation of Toll-like or TNF receptors, ERK signaling is activated and induces expression of IL-10, a key anti-inflammatory cytokine. We have found that monocytes from MPN patients have elevated p38MAPK activation and delayed TNFα production following TLR stimulation (22). While MPN monocytes produce IL-10, these cells are less responsive to IL-10 as measured by persistent phosphorylation of p38 and elevated intracellular TNFα levels (22). Furthermore, MPN monocytes had decreased STAT3 phosphorylation and suppressor of cytokine signaling 3 (SOCS3) gene expression in the presences of IL-10 compared to monocytes from a healthy patient and blocking IL-10 in normal cells phenocopies MPN monocytes (22). What remains to be understood is whether JAK2 mutant HSCs have a selective advantage in the presence of inflammation in the context of dampened IL-10 signaling.

One approach to study human HSC response to inflammation in MPN versus non-MPN patients involved single cell RNA-sequencing in stimulated versus unstimulated cells (data not shown). We can observe the upregulated versus downregulated gene pathways in cells from an MPN patient and non-MPN patient to gain insights on the dysregulated pathways in MPN cells. Our hypothesis is that overexpression of NF-κB-related genes in MPN HSCs will promote survival rather than apoptosis in response to TNFα-induced inflammation compared to non-MPN HSCs.

This hypothesis is based on our previous findings that JAK2^{V617F} cells have increased survival in the presence of TNFα (23). This experiment is ongoing, and additional samples are required to make general statements about MPN stem cell biology in the context of inflammation.

Therapeutic and/or lifestyle interventions aimed at reducing chronic inflammation could have marked effects on the many complications that MPN patients experience, which include thrombosis, fibrosis, heart disease, sepsis and bone marrow failure (24). Given NAC's multiple anti-inflammatory effects in vivo, this drug is attractive for the MPN patient population that exhibit a host of complications driven by inflammation. However, we did not identify a clear mechanism of how NAC prevents thrombosis in MPN and as such, it is difficult to identify an experimental readout that would determine NAC's effectiveness. This is one challenge that must be overcome if this drug is to be considered for clinical trials, which requires dose escalation and dose selection. Furthermore, long term treatment with antioxidants has been shown to increase cancer incidence. For instance, daily supplementation of beta-carotene increases the incidence of lung cancer and death in smokers (25-26). Daily supplementation of vitamin E was also found to increase prostate cancer incidence in men ages 50 and over (27). Thus, it appears that dietary antioxidant supplementation increases cancer progression if pre-malignant cells are present (such as in smokers) but does not increase cancer incidence in young or otherwise healthy individuals.

Driving apoptosis or inducing exhaustion may be one viable therapeutic option to target JAK2 mutant cells. Driving exhaustion through accelerated differentiation is the current theory of how interferon alpha therapy reduces mutation burden in MPN patients. However, IFNα therapies cause considerable toxicities in patients so tolerable formulations are continuously being generated. The ideal therapy would spare non-mutant cells from stress or exhaustion while selectively killing JAK2^{V617F} cells. The SMAC mimetic LCL-161 showed promise in this regard

as it was able to potently induce cell death in JAK2 mutant cells in Chapter 4. This sensitivity to apoptosis was lost in the presence of TNFα which induced cell death in wildtype and JAK2 mutant cells alike. Interestingly, inhibition of JAK2 or NF-κB increased viability of $JAK2^{V617F}$ cell line to LCL-161, suggesting that NF-κB is not protective for $JAK2^{V617F}$ cell survival rather that NF-κB activation is driving the sensitivity to cell death. Our data suggests that a bad therapeutic combination would include a JAK inhibitor such as Ruxolitinib with a SMAC mimetic, or an NF-κB inhibitor with a SMAC mimetic. In conclusion, ideal therapies would reduce TNFα and drive apoptosis in JAK2 mutant cells at doses that are tolerable for normal cells.

In conclusion, we revealed new insights regarding hematopoietic stem cell responses to inflammation. The $JAK2^{V617F}$ mutation drives cell cycling and differentiation of blood-forming stem cells and this phenotype was dampened following transplantation into a wildtype host. These data suggest that the bone marrow microenvironment plays an important role in HSC response to inflammation, and it remains to be seen whether this environment could contribute to replication stress, mutational burden and disease progression. This is a plausible theory given that different strains of mice develop different types of MPN; for example, mice of the Balb/C strain can develop myelofibrosis while C57Bl/6 develop polycythemia vera-type disease. In order to understand MPN etiology, these mechanisms would need to be well-defined in future studies. Ultimately, therapies aimed to reduce inflammation would have multiple benefits for MPN patients by reducing symptom burden and possibly preserving the fitness of normal blood forming stem cells to impede further clonal hematopoiesis.

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