UNIVERSITY OF CALIFORNIA,
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Investigating Therapeutics to Inhibit Hematopoietic Stem Cell Proliferation in Response to Inflammatory Stress

THESIS

submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in Biomedical and Translational Science

by

Sultan A. Alsuhaibani

Thesis Committee:
Professor Angela G. Fleischman, Chair
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Professor Sheldon Greenfield

2023

DEDICATION

To

my parents Abdullah and Norah, brother, sisters, colleagues, and friends

to Imagination

"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution."

Albert Einstein

to critical thinking and problem solving

"One of the first things one should know in order to understand the nature is that the knowledge of any thing which is sought can be acquired in no way except through the knowledge of its causes and principles."

Ibn Sina (Avicenna)

and to adventurousness

"Stay Hungry Stay Foolish"

Steve Jobs

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LIST OF ABBREVIATIONS

MPN	: Myeloproliferative Neoplasms
RBCs	: Red Blood Cells
WBCs	: White Blood Cells
HSCs	: Hematopoietic Stem Cells
JAK2	: Janus Kinase 2
CALR	: Calreticulin
TpoR	: Thrombopoietin Receptor
MPL	: Myeloproliferative Leukemia Virus Oncogene
ET	: Essential Thrombocythemia
PV	: Polycythemia Vera
PMF	: Primary Myelofibrosis
TNF-α	: Necrosis Factor Alpha
IL-6	: Interleukin-6
IL-10	: Interleukin-10
IL-1β	: Interleukin-1 beta
TET2	: Tet Methylcytosine Dioxygenase 2
DNMT3A	: DNA Methyltransferase 3 Alpha
ROS	: Reactive Oxygen Species
WT	: Wildtype
IACUC	: Institutional Animal Care and Use Committee
N-AC	: N-Acetylcysteine
LPS	: Lipopolysaccharide
IP	: Intraperitoneal

ACK	: Ammonium-chloride-potassium
FBS	: Fetal Bovine Serum
PBS	: Phosphate-buffered-saline
CD	: Cluster of Differentiation
CM-H2DCFDA – DCF	: chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate
DAPI	: 4′,6-diamidino-2-phenylindole
UCI	: University of California, Irvine
SEM	: Standard Error of the Mean
ANOVA	: Analysis of Variance
Lin^-	: Lineage negative
c-Kit	: c-Kit stands for "cellular Kit," where "Kit" is derived from "kitten," a name associated with the disease caused by the related viral oncogene, v-Kit, in cats. Where, cells expressing both stem cell factor receptor
Sca-1	: Stem Cell Antigen
HSPCs	: Hematopoietic Stem and Progenitor Cells
LKS	: Lineage negative, c-Kit positive and Sca-1 positive
SLAM	: Signaling Lymphocyte Activation Molecule, which identify cells expressing CD150 but not expressing CD48
LT-HSCs	: Long Term – Hematopoietic Stem Cells
MFI	: Mean Fluorescence Intensity
G phase	: Gap 0, 1 & 2
S phase	: DNA Synthesis
M phase	: Mitosis and cytokinesis
NETs	: Neutrophil Extracellular Traps

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ABSTRACT OF THE THESIS

Investigating Therapeutics to Inhibit Hematopoietic Stem Cell Proliferation in Response to Inflammatory Stress

by

Sultan A. Alsuhaibani

Master of Science of Biomedical and Translational Sciences

University of California, Irvine, 2023

Professor Angela Fleischman, Chair

Importance: Hematopoietic stem cells (HSCs) are foundational to the body's ability to mount an effective immune response, especially when confronted with acute inflammatory stress. Their role in rapidly increasing the number of mature effector leukocytes is essential for effective host defense. In the realm of hematological disorders, Myeloproliferative neoplasm (MPN) presents a significant challenge, primarily due to its effect on HSC function and proliferation.

Objective: Our laboratory previously showed that the antioxidant N-acetylcysteine (N-AC) might have a pivotal role in extending the lifespan of a Jak2V617F knock-in mouse model of MPN. This led to speculations about its protective effects on HSCs, especially against chronic inflammation. This study was consequently formulated with the primary aim to uncover the intricate mechanism by which N-AC shields HSCs from excessive proliferation when faced with inflammation.

Methods: We developed a protocol to examine the cell cycle status of HSCs, particularly after stimulation with lipopolysaccharide (LPS) — a known inflammatory agent. Mice were assigned into two groups randomly: one group received N-AC in their drinking water over a span of ten days, while the control group was given regular water. Mice were injected intra-peritoneally (IP) with lipopolysaccharide (LPS) at 1μg/mouse, and subsequently euthanized 16 hours later. Bone marrow was harvested from the four long bones of the hind limbs. Thereafter, staining procedures were employed to identify HSC-specific surface markers. Advanced techniques, involving the nuclear antigen, Ki67, and the DNA binding dye, DAPI, were used to determine cell cycling.

Results: The inflammation triggered by LPS led to a marked elevation in HSC division, a fact underscored by the surge in the percentage of cells in the G1 phase of the cell cycle. However, a notable observation was that the N-AC administered group exhibited a significant attenuation in this LPS-induced HSC cycling.

Conclusion and Relevance: Our findings illuminate the protective potential of N-AC against inflammation-induced exhaustion in HSCs. With HSCs being fundamental to hematological health, the therapeutic implications of N-AC, particularly for patients grappling with MPN, are profound. This could potentially pave the way for novel therapeutic strategies aimed at preserving the vital functions of HSCs in such patients.

I. INTRODUCTION

Myeloproliferative Neoplasms (MPN)

Myeloproliferative neoplasms (MPN) are hematologic malignancies characterized by overproduction of red blood cells (RBCs), white blood cells (WBCs) or platelets. BCR-ABL-negative or Philadelphia chromosome-negative MPNs arise from mutations in hematopoietic stem cells (HSCs) in the bone marrow resulting in abnormal cell proliferation [1]. The most common somatic mutations found in MPN occur in the Janus kinase 2 (JAK2) [2,3], calreticulin (CALR) [4] and thrombopoietin receptor (TPOR or MPL) [5] genes. The Jak2 activating mutation is an acquired point mutation, 1849G>T, resulting in an amino acid change from valine to phenylalanine at position 617 (V617F). The Jak2V617F mutation leads to cytokine independent activation of growth factor receptors such as the thrombopoietin receptor (TpoR) and erythropoietin receptor (EpoR), resulting in the abnormal production of red blood cells, white blood cells and platelets. Classical Philadelphia chromosome-negative (Ph-) myeloproliferative neoplasms (MPN) constitute a collection of clonal myeloid stem cell disorders, encompassing essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) [6–8]. The JAK2 V617F mutation is found in over 95% of those with PV and roughly 50% of those diagnosed with ET and PMF [9].

Inflammation and Myeloproliferative Neoplasms

Inflammation is a pathophysiological response generally elicited by external stimuli, including physical or chemical trauma, radiation exposure, or infection. Moreover, chronic hypoxia, as observed when cells aggregate in a solid tumor, within the bone marrow in the setting of hematological malignancy, or in any tissue affected by venous or arterial thrombosis, can also induce inflammation [10–12].

Chronic inflammation, a hallmark feature of myeloproliferative neoplasms (MPN), plays a pivotal role in the disease's initiation, progression, and symptomatology.[13,14]. Myeloid malignancies are typified by the presence of heightened levels of inflammatory cytokines, a phenomenon that is associated with the onset and progression of the disease, as well as the symptomatic burden and overall prognosis. In patients with myeloproliferative neoplasms (MPN), elevated plasma levels of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), lipocalin-2, and interleukin-6 (IL-6), have been consistently reported.
[15–17]. In addition to murine models of myeloproliferative neoplasms (MPN) [18].

Activation of the JAK/STAT signaling pathway by oncogenic MPN mutations enhances inflammatory cytokine production. Prior history of autoimmune diseases also increases the risk for MPN suggesting that an inflammatory milieu itself can lead to MPN [19]. Current treatments for MPN include anti-inflammatory and immunosuppressive drugs, such as JAK inhibitors, corticosteroids and interferon-α, to relieve symptomatic burden in MPN patients, prevent complications and halt disease progression.

Inflammation plays a critical role in the pathogenesis of MPN by promoting the growth of the mutant clone while suppressing the survival of the normal cells. Fleischman et al, demonstrated that an inflammatory environment consisting of TNF-α favors the expansion of the JAK2V617F mutant clones from MPN patients while normal or wildtype progenitor cells from MPN patients were hypersensitive to TNF-α mediate growth suppression [20]. Recent research from the Fleischman lab shows that blockade of the anti-inflammatory interleukin-10 (IL-10) pathway results in an expansion of the Jak2V617F mutant cells in a mouse model of MPN (unpublished data). Inflammatory stress also encourages the selective outgrowth of other hematopoietic mutations such as in the Tet methylcytosine dioxygenase 2 (Tet2) [21] and DNA methyltransferase 3 alpha (Dnmt3a) [22] genes. Thus, inflammation plays a role in the expansion of specific mutant hematopoietic clones. In the presence of inflammation, wildtype or normal HSCs are forced to proliferate and deplete while mutant hematopoietic cells evade these cues through different mechanisms and gain a selective growth advantage (Figure 1).

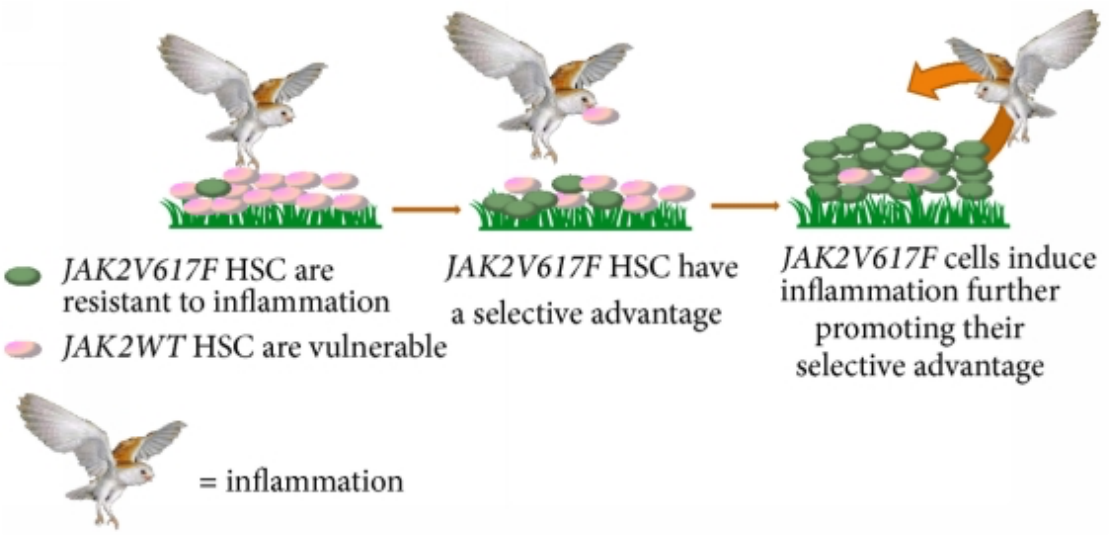

Figure 1. Inflammation as a driver of clonal expansion [23]. Jak2V617F-mutant HSCs are resistant inflammation while wildtype HSCs succumb to the deleterious effects of elevated inflammation. This endows Jak2V617F-mutant HSCs with a selective growth advantage.

An elevated inflammatory state is also associated with an altered redox balance. Reactive oxygen species (ROS) participate in cellular homeostasis but are also prime offenders contributing to disease pathogenesis. ROS play a causative role in HSC dysfunction due to exit from quiescence and proliferative stem cell exhaustion [24] (Figure 2).

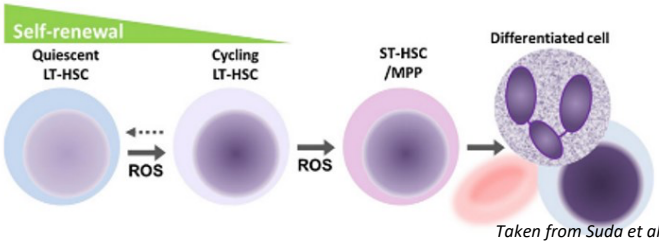

Figure 2. Reactive oxygen species (ROS) induces HSC division. Long-term HSCs (LT-HSCs) remain quiescent until stimulated to divide. Cycling and terminal differentiation is induced by increased reactive oxygen species.

We hypothesized that reducing oxidative stress will prevent proliferation and preserve the WT HSC compartment. N-acetylcysteine (N-AC) is an antioxidant involved in glutathione replenishment and is a potent free radical scavenger with anti-inflammatory properties. N-AC is clinically used as an anti-dote in acetaminophen poisoning. Due to its mucolytic action, N-AC is also used in cystic fibrosis. In addition, there are several research studies that provide evidence on the anti-inflammatory properties of N-AC. We have previously demonstrated that N-AC can extend the lifespan of a Jak2V617F knock-in MPN mouse model by inhibiting thrombosis [25]. N-AC has also been shown to improve human HSC engraftment in immunocompromised mice by decreasing reactive oxygen species in the bone marrow [26].

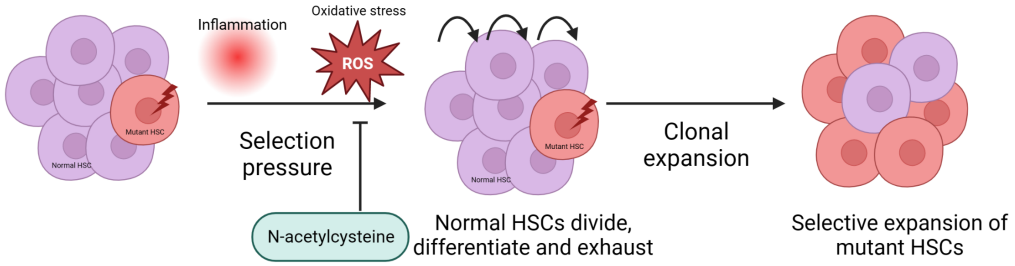

Figure 3. Model of clonal expansion induced by oxidative stress. We hypothesize that N-acetylcysteine, through mitigation of oxidative stress, can limit normal HSC proliferation and thus maintain their survival.

In this project, we tested the hypothesis that N-AC can inhibit proliferation of murine HSCs following an inflammatory stimulus (Figure 3).

Histone 2B-GFP Mice

To develop a powerful and sensitive tool for in vivo cell cycle analysis, it is imperative to adopt a strategy that entails the fluorescent labeling of proteins, which inherently localize to chromosomes within living cells. The nucleosome serves as the basic recurrent structure of chromatin. Each nucleosome core particle is composed of a histone octamer, around which 146 base pairs of micrococcal-nuclease-resistant DNA are coiled [27]. Given that histones are the primary structural proteins in eukaryotic chromosomes, they present as appealing candidates for fluorescent tagging. Rhodamine-conjugated histones from purified calf thymus (specifically H2A and H2B) have been microinjected into Drosophila embryos to study cellular lineage connections [28] and the processes of chromosomal condensation and decondensation [29]. The effectiveness of this method underscores the value of using fluorescently tagged histones to explore chromosomal movements within live cells. The green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* maintains its fluorescence when the recombinant GFP proteins are produced in eukaryotic cells. GFP fusion proteins have been effectively directed to particular subcellular compartments and formations such as the nucleus, plasma membrane, mitochondria, cytoskeleton, and Golgi apparatus [30,31]. Furthermore, tagging with GFP has facilitated the observation of distinct chromosomal areas [32–34]. These findings suggest the promising application of a histone-GFP fusion protein for fluorescent chromosome marking in live cells. The viability of this method is evident from the noted localization of a fusion protein, combining GFP and yeast histone H2B, within yeast nuclei [35]. The fusion protein of H2B-GFP integrates into nucleosomes without influencing cell cycle progression [27]. Consequently, histone 2B-GFP mice are a genetically engineered mouse model in which the

histone protein H2B is fused with the green fluorescent protein (GFP). Thus, the H2B-GFP fusion allows for real-time visualization of chromatin dynamics and cellular processes. Since histones are most notably involved in DNA packaging and regulation, their visualization offers a unique window into processes such as cell division, differentiation, and migration in living organisms. Moreover, Considering the fundamental importance of histones in DNA replication and chromosome segregation, these mice are invaluable for studies related to cell cycle progression and regulation, which is the focus of this project.Expression of the H2B-GFP fusion protein can be induced by doxycycline [36]. When treated with doxycycline, these mice have the drug binding to the tetracycline transactivator protein (tTA) complex, which then detaches from the tetracycline-responsive element (TRE), leading to the cessation of GFP expression. As cells divide, GFP expression diminishes, leaving only the dormant cells with a long-term GFP label [37].

N-Acetylcysteine

N-acetylcysteine (N-AC) functions as a potent antioxidant, exerting its effects through multiple mechanisms. First, N-AC exhibits a direct impact on specific oxidant species, mitigating their harmful effects. Second, it serves as an indirect antioxidant by acting as a precursor to cysteine, an essential amino acid required for the synthesis of glutathione, a key cellular antioxidant. Furthermore, N-AC possesses the ability to replenish thiol pools, which subsequently regulate the cellular redox state [38].

Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) refer to a class of highly reactive, unstable molecules derived from oxygen that are generated as secondary products during standard metabolic reactions [39]. Reactive Oxygen Species (ROS) mainly include unstable molecules like superoxide anion, singlet oxygen, hydrogen peroxide, hypochlorous acid, and hydroxyl radical. Their reactivity comes from extra unpaired electrons, and they have a very short half-life, measured in milliseconds [40]. Numerous studies have highlighted the dual role of Reactive Oxygen Species (ROS) in cancer biology, acting either as tumor suppressors or promoters. Specifically, ROS is regarded as a tumor-suppressing agent when its production is elicited by chemotherapeutic agents. This action is attributable to ROS's role in inducing cell death, a common mechanism exploited by most chemotherapy treatments [41]. Nevertheless, certain species of reactive oxygen species (ROS) have the potential to influence diverse cellular functions, including cell proliferation. In the context of neoplastic cells, ROS are commonly considered oncogenic due to their association with the initiation, progression, and metastasis of tumorous growths [42].

Specific Aims

The present study is informed by recent evidence highlighting the potential efficacy of N-acetylcysteine (N-AC) in inhibiting inflammation. Prior work has demonstrated that N-AC can prolong the lifespan of a Jak2V617F knock-in MPN mouse model by inhibiting thrombosis [25]. N-AC has also been found to enhance human HSC engraftment in immunocompromised mice by decreasing reactive oxygen species in the bone marrow [26]. In this experimental investigation, we will pursue the following specific aims:

Primary Aim: To establish a robust and reproducible method for assessing hematopoietic stem cell (HSC) proliferation in response to inflammatory stimuli.

Inflammatory Stimulus: Acute inflammatory stress in mice will be induced using lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls.

Assessment of HSC Division: Two primary techniques will be utilized:

- **Ki67 and DAPI Staining:** Ki67 acts as a marker for proliferating cells. When used alongside DAPI, a DNA stain, it can effectively discern the cell cycle status of different cell types. This methodology will be employed to fulfill the secondary aim of my research.
- **TetOP-H2B-GFP Mice:** Mice colony has been established in the Fleischman lab., this mouse model demonstrates doxycycline-induced GFP expression across a spectrum of cell types. Upon the induction of GFP expression via doxycycline water, an LPS stimulation will be introduced. The consequent rate of GFP protein depletion from long-term HSCs (LT-HSCs) will be instrumental in analyzing divisional history.

Secondary Aim: Investigate the potential protective effects of N-acetylcysteine (N-AC), a renowned antioxidant and anti-inflammatory agent, on normal stem cells subjected to inflammation-induced proliferation. The specific objectives under this aim are:

- **Elucidation of N-AC's Role in HSCs:** Assess the ability of N-acetylcysteine (N-AC) to mitigate oxidative stress in hematopoietic stem cells (HSCs). I hypothesize that N-AC will effectively curtail oxidative stress in HSCs, enhancing their functionality and viability.
- **Evaluation of N-AC's Impact on HSC Proliferation:** Explore the efficacy of N-AC in attenuating HSC proliferation when exposed to acute inflammatory stimuli. I postulate that the intervention with N-AC could diminish the proliferation of HSCs in inflammatory settings.

Understanding and harnessing such a response not only holds profound implications for the preservation of stem cell function but also offers a promising avenue for therapeutic interventions. The ultimate goal is to mitigate or even impede the progression of disorders, particularly in patients diagnosed with myeloproliferative neoplasm (MPN). This could redefine current approaches to stem cell-related diseases and foster advancements in targeted treatment modalities.

II. METHODS

Mice

For all experimental procedures, C57BL/6J mice of wildtype (WT) lineage, aged between 8-10 weeks, were utilized. Conversely, Histone 2B-GFP mice, within the same age bracket, were specifically employed to assess HSC proliferation. Mice were housed in specific pathogen-free facilities at the University of California, Irvine and maintained on a 12-hour light/dark cycle. All animal procedures were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine.

Acute Inflammation Model

Mice were randomly assigned to control acidified water or N-acetylcysteine water groups. N-acetylcysteine (N-AC) (MilliporeSigma), was dissolved in deionized water at 2g/L and was administered to mice as their drinking water. N-AC water was replaced every other day for ten days. To induce an acute inflammatory hematopoietic stress response, mice were injected intra-peritoneally (IP) with lipopolysaccharide (LPS) from Escherichia coli (MilliporeSigma) at 1μg/mouse. Control mice received phosphate buffered saline, IP.

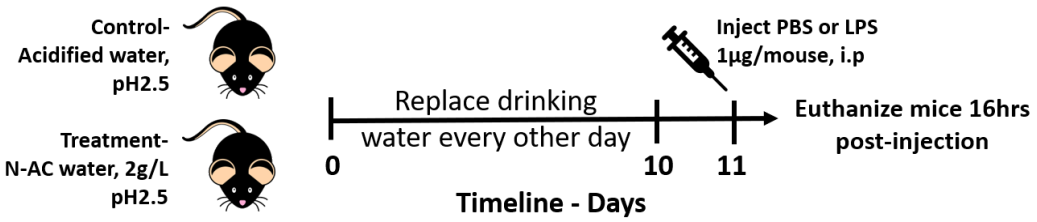

Figure 4. Experimental design. Mice were provided N-acetylcysteine in the drinking water and acute inflammation was induced by intra-peritoneal (IP) injection with lipopolysaccharide (LPS). The experiment consisted of 3-4 mice per condition and the experiment was repeated at least twice.

Mouse Bone Marrow Isolation

Mice were euthanized 16 hours after the LPS injection and bone marrow was harvested from the long bones of the hind limbs. Bone marrow was directly flushed onto a 40µM cell strainer using a 27-guage needle attached to a 10ml syringe containing phosphate buffered saline with 2% fetal bovine serum (2% FBS/PBS or staining buffer). Cells were collected by centrifugation at 400 x g for 10 minutes. Red blood cells were lysed by incubating with ammonium chloride-potassium (ACK) buffer for 10 minutes on ice. Bone marrow cells were then washed by adding 15ml of staining buffer, centrifuging at 400 x g and finally resuspended in 1ml of staining buffer for labeling of HSCs.

Flow Cytometry Analysis of Mouse Bone Marrow

All antibodies were purchased from BioLegend unless otherwise stated. Bone marrow hematopoietic cells were stained with Pacific blue-conjugated anti-mouse antibodies against TER-119 (clone Ter119), CD3 (clone 17A2), Gr-1 (clone RB6-8C5), CD11b (clone M1/70) and B220 (RA3-6B2) to detect mature cells (Lineage cocktail). Ter119 is a surface marker on erythroid cells, CD3 is expressed on T lymphocytes, Gr-1 and CD11b are used to label cells of the myeloid lineage such as monocytes, neutrophils, and eosinophils while B220 is a pan mouse B cell marker. HSC populations were detected by staining with c-Kit (clone 2B8), Sca-1 (clone D7), CD48 (clone HM48-1) and CD150 (clone TC15-12F12.2). Cells were incubated with the above antibodies at 1/100 dilution in staining buffer for 30 minutes at 4°C. Cells were then washed and processed for detection of oxidative stress and assessment of cell proliferation. The staining scheme employed was as follows: Lineage negative (comprising anti-mouse Ly6G/Ly6C, CD3, CD1b, B220, Ter-119), c-Kit positive, Sca-1 positive, CD48 negative, and CD150 (SLAM) positive. These markers identify what are commonly referred to as LKS-SLAM cells (Figure 1).

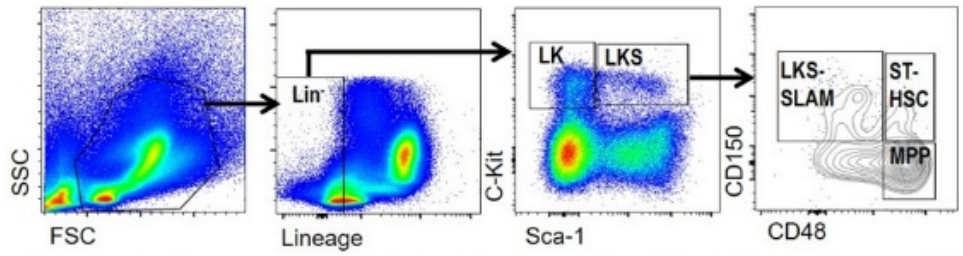

Figure 5. **Staining scheme used to identify long-term mouse HSCs or LKS-SLAM cells from bone marrow.** ST-HSC, short-term HSC; MPP, multipotent progenitor.

Detection of Reactive Oxygen Species

For the detection of reactive oxygen species in HSCs, the chloromethyl derivative of 2'7'-di-chlorofluorescein (CM-H2DCFDA) (ThermoFisher Scientific) was used. DCF-DA is a cell permeant indicator for reactive oxygen species. Oxidation of DCF within the cell leads to increased fluorescence that can be detected by flow cytometry. Surface-stained bone marrow cells were incubated with 5μM of CM-H2DCFDA in plain cell culture media for 30 minutes at 37°C. Cells were then washed with phosphate buffered saline (PBS) and analyzed by flow cytometry.

Assessment of Cell Proliferation

To evaluate cell proliferation, I employed two distinct methods

1. **Ki67 and DAPI Staining:** surface-stained bone marrow cells were fixed and permeabilized for 30 minutes on ice using the BD cytofix/cytoperm buffer (BD Biosciences). Cells were washed with 1X BD perm/wash buffer and permeabilized with the BD permeabilization buffer plus. Cells were washed with 1X BD perm/wash buffer and re-fixed with BD cytofix/cytoperm buffer for 5 minutes on ice. Anti-mouse Ki67 antibody (clone 16A8) was diluted 1/50 in 1X BD perm/wash buffer and incubated with the fixed and permeabilized cells for 20 minutes at room temperature in the dark. Cells were then washed with staining buffer and DNA was stained using 4',6-diamidino-2-phenylindole, dilactate (DAPI) (ThermoFisher Scientific) at a final concentration of 1μg/ml diluted in phosphate buffered saline. Cells were incubated with DAPI for 10 minutes at room temperature and washed with staining buffer.

2. **TetOP-H2B-GFP Mice:** Bone marrow cells, post surface-staining, were managed with care to maintain integrity. They were kept on ice and shielded from light to ensure the preservation of GFP fluorescence. This was followed by a wash with the designated staining buffer.

For both aforementioned methodologies, flow cytometry data acquisition transpired on the Novocyte (ACEA Biosciences) housed at the UCI Immunology Core facility. Data analysis was facilitated through the FlowJo™ software (BD Biosciences)."

Statistical Methods and Analysis

Statistical evaluations were conducted utilizing GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data are displayed as means, and the corresponding error bars denote the standard error of the mean (SEM). To assess the statistical significance of differences between two groups, an unpaired Student's t-test was employed. In instances where more than two groups were being compared, a two-way analysis of variance (ANOVA) was implemented, followed by a post-hoc correction for multiple t-tests using Bonferroni's method. All statistical significance was determined based on the adjusted p-values.

II. RESULTS

N-Acetylcysteine Reduces Oxidative Stress in HSCs

Wildtype C57BL/6J mice were administered N-AC in their drinking water and challenged with LPS to induce an acute inflammatory response that is associated with stress hematopoiesis. Murine bone marrow long term-HSCs or LKS-SLAM HSCs were identified using specific antibodies binding to cell surface antigens and a well-established gating strategy (Figure 6A). Briefly, first live cells are gated using the forward and side scatter plot. Then, lineage negative (Lin^-) cells are identified because cells expressing any of the lineage specific markers are mature cells. The lineage negative gate consists of immature cells that includes both stem, progenitor and committed progenitor cells. From the lineage negative population, cells expressing both stem cell factor receptor (c-Kit) and stem cell antigen-1 (Sca-1) were identified as hematopoietic stem and progenitor cells (HSPCs). Since, these HSPCs are lineage negative, c-kit positive and Sca-1 positive, these cells are termed the "LKS" population. Of the LKS population, we then identify cells expressing CD150 or signaling lymphocyte activation molecule (SLAM) marker but not expressing CD48 since quiescent long-term HSCs exclude CD48 surface antigen. These cells represent true long-term HSCs and are termed LKS-SLAM since they express the CD150 or SLAM marker. The LT-HSCs are Lin^-, c-Kit^+, Sca-1^+, $CD150^+$ and $CD48^-$.

As expected, LPS challenge induced a strong increase in Sca-1 expression on hematopoietic stem and progenitor cells (HSPCs) (Figure 6A, lower panel). This indicates that the mice that received LPS exhibited characteristic inflammatory responses. LPS challenge at 1μg also significantly increased reactive oxygen species in bone marrow HSCs while N-AC supplementation was able to suppress LPS induced oxidative stress in HSCs as determined by the DCF fluorescence intensity (Figure 6B). LPS also induced a distinct DCF positive population in the HSC compartment of mice receiving acidified water (Figure 6B). However, compared to the control group, the N-AC group displayed a significantly reduced percentage of DCF positive HSCs (Figure 6B). These observations demonstrate that N-AC supplementation is effective at diminishing oxidative stress in HSCs under conditions of inflammatory stress.

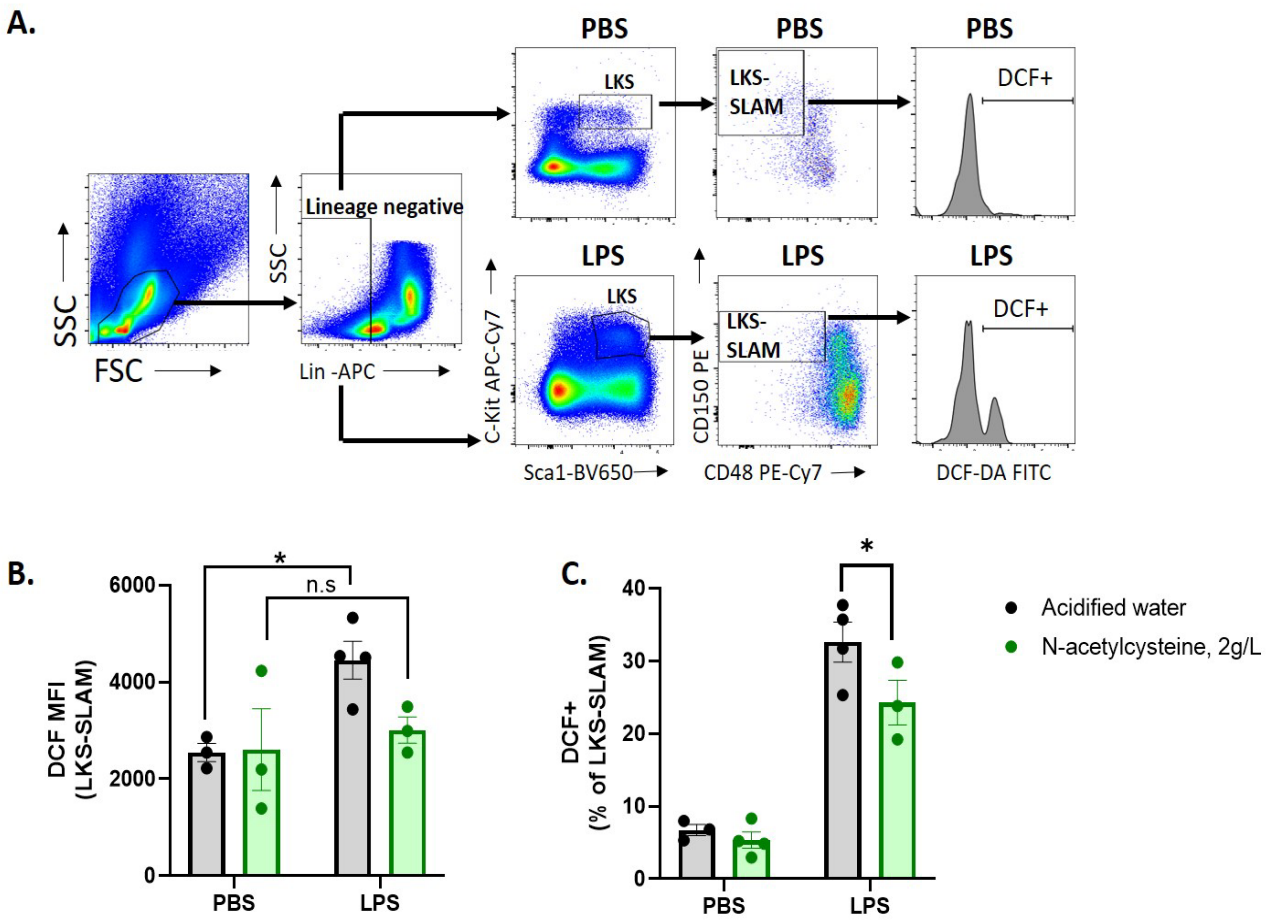

Figure 6. N-acetylcysteine decreases ROS in LT-HSCs. (A) Gating scheme used to identify LT-HSCs or LKS-SLAM HSCs by flow cytometry. (B) Lipopolysaccharide (LPS) injection induces increased ROS in HSCs compared to control PBS mice, while N-AC prevents LPS-induced oxidative stress as measured by mean fluorescence intensity (MFI) of the DCF-DA dye. (C) N-AC decreases the DCF positive HSC population compared to the control group. LKS, lin$^-$ cKit$^+$ Sca1$^+$; LKS-SLAM, lin$^-$ c-Kit$^+$ Sca1$^+$- CD150$^+$ CD48$^-$. PBS, phosphate buffered saline. Data are shown as mean±SEM. *p<0.05, Unpaired Student's t-test.

N-Acetylcysteine Reduces HSC Division

To determine if the N-AC mediated reduction in oxidative stress following LPS inflammation results in diminished HSC division, we assessed cell cycle status by staining cells with Ki67 and DAPI. Ki67 is a widely used proliferation marker and is an antigen detected within the nucleus during the different phases of the cell cycle but is completely absent during the resting or quiescent phase (G0). DAPI is a DNA stain that has high affinity for double stranded DNA and is used to stain DNA in fixed and permeabilized cells since it is cell impermeant.

Robust Ki67 signal was able to distinguish between resting (G0) and dividing (G1) cell populations (Figure 7A). By assessing DAPI incorporation, G2, S and mitotic phases of the cell cycle were also identified in LPS challenged mice (Figure 7A).

In mice that received acidified water, LPS injection induced a significant increase in the percentage of dividing cells (G1 phase) when compared to the PBS control group, as observed by the enhanced expression of Ki67 in LT-HSCs (Figure 7B). Interestingly, mice that received N-AC supplementation did not exhibit elevated HSC cycling when challenged with LPS (Figure 7B). The percentage of dividing HSCs was also significantly different between the acidified water and N-AC LPS groups (Figure 7B). Increased intra-cellular Ki67 following LPS stimulation was also accompanied by increased DAPI signal suggesting indicative of different phases of the cell cycle (Figure 7A). Our data demonstrates that N-AC can limit HSC proliferation following an acute inflammatory stress by its ability to reduced intracellular oxidative stress.

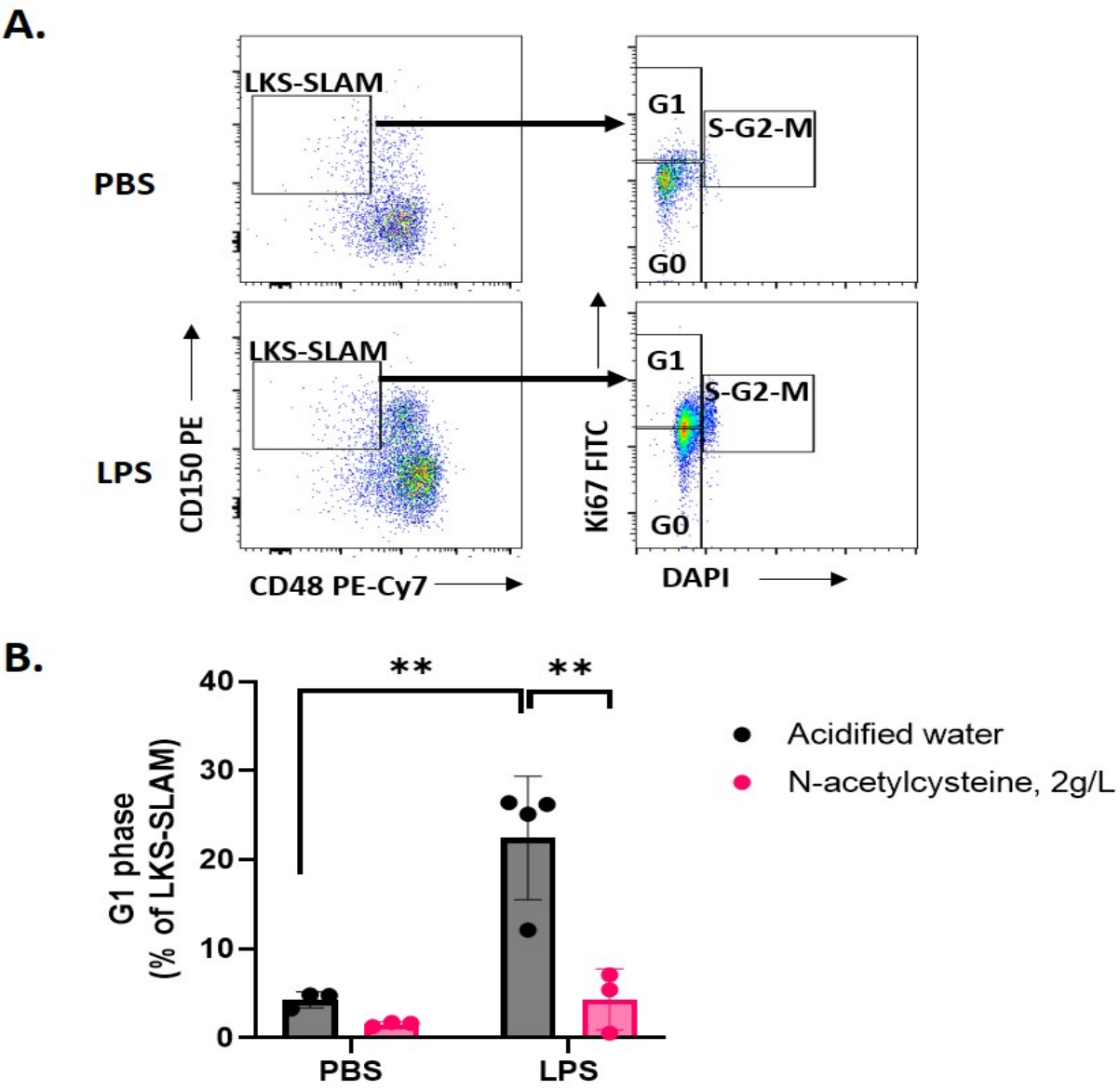

Figure 7. N-acetylcysteine limits HSC proliferation induced by acute inflammatory stress. (A) Staining scheme of LKS-SLAM HSCs to identify cycling cells using the proliferation antigen, Ki67, and DNA binding dye, DAPI. (B) Lipopolysaccharide (LPS) increases the percentage of HSCs in the G1 phase of cell cycle in control mice while mice drinking N-AC water displayed decreased HSC division in the presence of LPS. Data are shown as mean±SEM. **p<0.01, Unpaired student's t-test.

The TetOP-H2B-GFP Mouse Model:

An Efficacious Approach to Assess Cell Division.

To evaluate the efficacy of the TetOP-H2B-GFP Mouse Model in assessing cell division, I utilized the H2B-GFP expression in tandem with established surface markers, notably lineage markers (L), c-Kit (K), Sca-1 (S), CD48, and CD150 (refer to figure 5). By doing so, I compared proliferation rates of specified populations through the observation of H2B-GFP retention over time. Within the TetOP-H2B-GFP Mice, the variable loss of the GFP signal served as a tool to differentiate resting (G0) from dividing (G1) cell populations. Upon subjecting these mice to an LPS challenge, there was a marked decline in the GFP signal during the cell cycle relative to the control group. This observation accentuates the precision and utility of the TetOP-H2B-GFP system in gauging HSC division.

IV. DISCUSSION

Assessment of Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are characterized by their ability to self-renew and differentiate to give rise to all blood lineages throughout life. Under steady-state conditions, most HSCs are dormant or quiescent and hematopoiesis is maintained through a few dividing HSCs. However, external triggers can stimulate HSCs to proliferate and differentiate to increase the output of mature effector cells, also known as stress-hematopoiesis. Stress signals that induce rapid HSC proliferation include infection, inflammation, excessive blood loss, and chemotherapy. Reactive oxygen species (ROS) are potent inducers of HSC exit from quiescence, repeated cell division, differentiation, and accelerated exhaustion. ROS reduction in HSCs using N-acetylcysteine (N-AC) has shown to improve HSC reconstitution following bone marrow transplantation [43]. Investigating novel and feasible approaches that protect HSCs from continuous proliferative stress could lead to therapeutic opportunities to prevent accelerated wildtype HSC exhaustion MPN disorders.

Recent findings from our lab show that low-grade inflammation caused by interleukin-10 receptor blockade in a mouse model of MPN, results in the selective outgrowth of Jak2V617F cells compared to their normal counterparts. We speculate that inflammation induced oxidative stress drives wildtype HSCs to divide and exhaust while the mutant cells gain a selective advantage. In this project, we tested if N-AC can protect wildtype HSCs from inflammation induced cycling and exhaustion. We observed that N-AC can reduce ROS accumulation and inhibit proliferation in HSCs following acute inflammation.

Limitations

While the present study provides valuable insights, it is important to acknowledge certain limitations inherent to our experimental design and methodology. We used a single low dose of LPS in an to induce acute inflammation, but we did not test if N-AC can protect HSCs in a chronic inflammatory microenvironment. This will be relevant since MPN is characterized by a chronic inflammatory state.

N-AC was provided to the mice orally at a concentration of 2g/L equivalent to a 12mM solution. N-AC is known to have low bioavailability due to first pass metabolism and it is possible that the complete antioxidant effect of N-AC has not been captured in this experimental design.

The current experimental data was obtained from wildtype mice. However, we have not assessed the differential effect of N-AC, if any, on Jak2V617F mutant HSCs. We hypothesize that N-AC neutralizes the inflammatory microenvironment that provides mutant cells a growth advantage, thus limiting disease progression.

Although, we assessed oxidative stress and cell cycling of HSCs, we did not determine the anti-inflammatory effects exerted by N-AC upon LPS stimulation. To do this, we will determine plasma levels of pro-inflammatory cytokines such as TNF-α, interleukin-6 and interleukin-1β that signal for HSC division following LPS administration. This data will provide additional mechanisms through which N-AC limits stem cell division.
N-AC has already been shown to prevent thrombosis in MPN by inhibiting the formation of neutrophil extracellular traps (NETs) and platelet-leukocyte aggregates (PLAs) [25]. The current study also demonstrates that N-AC can be utilized to prevent wildtype stem cell exhaustion and could be a possible therapeutic in MPN.

Future Directions

Elucidating the Long-Term Impact of N-AC and LPS on HSCs: future studies will extend the duration of LPS administration in the presence of N-acetylcysteine (N-AC) and evaluate its sustained impact on HSC oxidative stress and proliferation. This will provide insights into the long-term effects of these agents on hematopoietic stem cells and their potential therapeutic implications.

Comparing Oral and Intra-Peritoneal (IP) N-AC Administration: subsequent investigations will compare the efficacy of oral versus intra-peritoneal (IP) administration of N-AC in modulating oxidative stress and stem cell proliferation. This will aid in determining the optimal route of administration for N-AC in the context of HSC modulation and inflammation.

Investigating the Role of N-AC in Mutant Mice: future research will employ mutant mouse models, rather than wild-type mice, to assess the capacity of N-AC to neutralize the inflammatory microenvironment that promotes mutant cell growth, thereby potentially mitigating disease progression. This will provide valuable information on the specific context in which N-AC may be therapeutically beneficial.

Examining Plasma Levels of Pro-Inflammatory Cytokines Following LPS Administration: we will measure plasma levels of pro-inflammatory cytokines, including TNF-α, interleukin-6, and interleukin-1β, that are known to stimulate HSC division following LPS administration. The data generated from these analyses will offer further insight into the mechanisms by which N-AC may restrict stem cell proliferation. This information will be critical for understanding the broader implications of N-AC administration on the inflammatory milieu and stem cell function.

V. SUMMARY AND CONCLUSIONS

In this study, we investigated the potential of N-acetylcysteine (N-AC) to protect wildtype hematopoietic stem cells (HSCs) from cycling and exhaustion induced by inflammation. Our observations revealed that N-AC effectively attenuated the accumulation of reactive oxygen species (ROS) and inhibited proliferation within HSCs in the context of acute inflammation. These findings highlight the potential therapeutic value of N-AC in safeguarding HSCs from the detrimental effects of inflammation-induced cycling and exhaustion. By reducing ROS accumulation and inhibiting HSC proliferation in the face of acute inflammation, N-AC may serve as an important protective agent for maintaining HSC viability and function. These observations justify further investigation of N-acetylcysteine (N-AC) as a potential therapeutic approach for alleviating inflammation-related hematopoietic aberrations, particularly in individuals with clonal hematopoietic mutations, and for impeding disease progression in patients with myeloproliferative neoplasms (MPN). Future studies should further elucidate the molecular mechanisms underlying the observed effects of N-AC and evaluate its potential clinical applications in the context of inflammation and hematopoietic disorders.

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