

UC Irvine

UC Irvine Electronic Theses and Dissertations

Title

Investigating the impact of combustible and electronic cigarettes on clonal hematopoiesis

Permalink

<https://escholarship.org/uc/item/5b38t262>

ISBN

9798270246969

Author

Sullivan, Jeanette

Publication Date

2026-12-24

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA,
IRVINE

Investigating the impact of combustible and electronic cigarettes on clonal hematopoiesis

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Jeanette Sullivan

Dissertation Committee:
Professor Angela Fleischman, Chair
Professor Peter Kaiser
Professor Marcus Seldin
Assistant Professor Minji Byun
Professor Wendy Liu

2025

TABLE OF CONTENTS

	Page
TABLE OF CONTENTS.....	ii
LIST OF FIGURES	iv
ACKNOWLEDGMENTS	vii
VITA.....	ix
ABSTRACT OF THE DISSERTATION	xi
CHAPTER ONE: The intersection of hematopoiesis, inflammation, and smoking.....	1
INTRODUCTION	2
1.1 HEMATOPOIETIC STEM CELLS	2
1.2 MYELOID CELLS	6
1.3. TRADITIONAL AND ELECTRONIC CIGARETTES	12
CONCLUSIONS: BRIDGING TOXICOLOGY AND HEMATOPOIESIS	16
REFERENCES	17
CHAPTER TWO: Cigarette Smoke and E-Cigarette Aerosol Extracts Induce Myelopoiesis and Suppress Inflammatory Cytokine Production.....	24
ABSTRACT.....	26
INTRODUCTION	27
MATERIALS AND METHODS.....	30
RESULTS	36
DISCUSSION.....	56
AUTHOR CONTRIBUTIONS.....	61
ACKNOWLEDGEMENTS.....	61
FUNDING.....	61

REFERENCES	62
CHAPTER THREE: Electronic cigarette inhalation promotes the expansion of JAK2V617F mutant hematopoietic stem cells.....	
ABSTRACT.....	69
INTRODUCTION	70
METHODS	72
RESULTS	76
DISCUSSION.....	83
REFERENCES	85
CHAPTER FOUR: Summary & Conclusions	
REFERENCES	95

LIST OF FIGURES

Figure 2.1. Cigarette smoke inhibits LPS-induced TNF- α secretion in multiple macrophage models.

Figure 2.2. Human PBMCs are diverse and display either a “stimulatory” or “inhibitory” response to cigarette smoke extract.

Figure 2.3. Cigarette smoke and e-cigarette extracts suppress hematopoietic progenitor function with partial resistance in Tet2-deficient cells.

Figure 2.4. Experimental setup of WT: Tet2^{-/-} competitive transplant with bone marrow co-cultured overnight in smoke or e-cigarette extract.

Figure 2.5. Exposure to CSE or EVE in HSCs induces long-term increases in circulating myeloid cell populations.

Figure 2.6. EVE exposure promotes the expansion of Tet2^{-/-} cells within the peripheral blood in a competitive transplant.

Figure 2.7. Impact of CSE and EVE on WT and Tet2^{-/-} monocytes.

Figure 2.8. Tet2^{-/-} chimerism in bone marrow at ten months post-competitive transplant with CSE and EVE-exposed HSCs.

Figure 2.9. Long-term inhalation of traditional and e-cigarette vape smoke increases the frequency of platelets and myeloid cells in the peripheral blood.

Figure 2.10. Exposure to smoke and e-cigarette vapor increases monocytosis and decreases neutrophil differentiation in a secondary transplant.

Figure 3.1. Experimental setup of WT:JAK2V617F and WT:WT transplants for *in vivo* e-cigarette exposure.

Figure 3.2. Mice exposed to E-cig aerosols demonstrated significant expansion of JAK2V617F cells at the end of the experiment compared to air-exposed controls.

Figure 3.3. Cytokine secretion in bone marrow-derived macrophages from mice exposed to air or e-cigarette aerosol.

Figure 3.4. P-selectin mean fluorescence intensity (MFI) following thrombin-stimulation on platelets.

Figure 3.5. Terminal bone marrow and spleen chimerism after e-cigarette exposure.

KEY ABBREVIATIONS

CSE – cigarette smoke extract

EVE – e-cigarette vape extract

HSC – hematopoietic stem cell

CH (or CHIP) – clonal hematopoiesis (of indeterminate potential)

BM – bone marrow

BMDM – bone marrow-derived macrophages

PB – peripheral blood

CBC – complete blood count

WT – wildtype

JAK – Janus kinase

TET – Ten-eleven translocation

TNF α – tumor necrosis factor alpha

JAK2V617F (or VF) – the most common mutation that causes MPN by converting valine into phenylalanine at the 617th amino acid of Janus kinase 2

LPS – lipopolysaccharide

ELISA – enzyme- linked immunosorbent assay

LKS – cell staining scheme to identify HSPCs (Lineage–ckit+Sca1+)

LKS-SLAM – cell staining scheme to identify HSCs (Lineage–ckit+Sca1+CD150+CD48–)

CFU – colony-forming unit

MPN – myeloproliferative neoplasm

MDS – myelodysplastic syndrome

ACKNOWLEDGMENTS

The PhD process is one of the most humbling yet empowering experiences; I have never felt so knowledgeable yet insignificant against the landscape of science. I am deeply grateful for all the support I have received in completing this dissertation and in preparing me to become a successful scientist. First, I would like to thank and acknowledge Dr. Angela Fleischman, my PI, for her guidance and support over the past four years. She believed in me even when I didn't believe in myself, and I am so grateful to have had the honor of being a part of the Fleischman Lab. I would not have made it through without my wonderful lab-mates. I would also like to thank Dr. Gaja Ramanathan, a postdoctoral fellow and specialist, whom I was fortunate to work with for the first two years of my PhD. I am forever grateful for her help in developing my e-cigarette research project. We are incredibly grateful for financial support from the University of California, Irvine Chao Family Comprehensive Cancer Center (CFCCC) and the Tobacco-Related Disease Research Program (TRDRP).

I am sincerely thankful to my committee members: Dr. Peter Kaiser, Dr. Marcus Seldin, Dr. Minji Byun, and Dr. Wendy Liu. Your insight and support have been invaluable, and I appreciate the time and knowledge you have given in my pursuit of a PhD. I want to thank and acknowledge Dr. Nicole R. Sparks and Nneamaka Iwobi from the Sparks Lab in the Department of Environmental and Occupational Health at the UC Irvine Joe C. Wen School of Public Health for their assistance in preparing the e-cigarette vapor extracts, particularly the wide variety of extract types we tested during troubleshooting. I also thank and acknowledge the Kleinman Lab (the Air Pollution Health Effects Laboratory, also of the Department of Environmental and Occupational Health at UCI), particularly Irene Hasen and Amanda Ting, for their assistance with the inhalation exposures.

Finally, I would like to thank all of my friends and family for their incredible support through the ups and downs of graduate school.

VITA
Jeanette Sullivan

EDUCATION

- 09/2021 – 12/2025 **Ph.D. in Biomedical Sciences**
University of California, Irvine, *Irvine, CA*
Dissertation: Investigating the impact of combustible and electronic cigarettes on clonal hematopoiesis
Advisor: Dr. Angela Fleischman
- 08/2016 - 01/2020 **B.S. in Biological Sciences**
Case Western Reserve University, *Cleveland, OH*
Magna cum laude
Honors thesis: Mechanisms of opioid regulation of human CD4+ T cells
Advisor: Dr. Alan D. Levine

EXPERIENCE

- 09/2021 – 12/2025 **Graduate Student Researcher**
University of California, Irvine, *Irvine, CA*
Skills: cell culture, multicolor flow cytometry, RNA isolation, qPCR, ELISA, murine primary cell isolation.
- 09/2020 – 08/2021 **Clinical Lab Technician**
Avellino Labs USA, *Menlo Park, CA*
Covid-19 testing: RNA extraction, PCR, results analysis, clinical reporting, high-throughput sample processing, workflow optimization.
- 12/2017 – 07/2020 **Undergrad Researcher**
Case Western Reserve University, *Cleveland, OH*
Analyzed how different opioid receptor agonists alter the immune response of T cells. Selected for prestigious undergraduate research award.
- 03/2017 – 03/2018 **Research Assistant**
National Prion Disease Pathology Surveillance Center, *Cleveland, OH*
Coordinated autopsies and neuropathologic examination of suspected prion disease cases.

PUBLICATIONS

Sullivan JYC, Fleischman AG. *Relating NF- κ B regulation to MPN pathogenesis*. Blood. 2024 Jun 6;143(23):2345-2347. doi: [10.1182/blood.2024024451](https://doi.org/10.1182/blood.2024024451). PMID: 38842861.

PROFESSIONAL AFFILIATIONS

- ❖ American Society of Hematology Member, Jun 2024-present

- ❖ Society of Toxicology Member, Feb 2024-present
 - Southern California Regional Chapter Member, Oct 2024-present
 - Graduate Student Representative, May 2025-2026

AWARDS & HONORS

- ❖ Honorable Mention, NSF GRFP 2020
- ❖ Arnold & Mabel Beckman Foundation Beckman Scholars Program, 2018-2019
- ❖ 1st place, CWRU Undergraduate Research Symposium Life Sciences Poster Competition, Dec 2019
- ❖ CWRU Junior-Senior Scholarship, Case Alumni Association, 2018
- ❖ University Merit Scholarship, CWRU 2016-2020

ABSTRACT OF THE DISSERTATION

Investigating the impact of combustible and electronic cigarettes on clonal hematopoiesis

by

Jeanette Sullivan

Doctor of Philosophy in Biological Chemistry

University of California, Irvine, 2025

Professor Angela Fleischman, Chair

Self-renewing hematopoietic stem cells (HSCs) produce billions of blood cells a day to maintain peripheral blood and immune cells in circulation. Inflammation can alter the balance of steady-state hematopoiesis, and disrupted hematopoiesis can lead to blood disorders or cancers. Over a lifetime of cell divisions, HSCs may acquire somatic mutations that provide a competitive advantage over their wild-type counterparts. The clonal expansion of a mutant HSC population is termed “clonal hematopoiesis” (CH). CH is linked to an increased overall risk of mortality due to incidences of cardiovascular disease and transformation into hematological malignancy. Tobacco and nicotine use remain the leading preventable drivers of cancer risk, and both direct and secondhand exposure to combustible cigarettes or electronic nicotine devices perturbs immune function and hematopoiesis. The World Health Organization estimates more than 100 million people across the world are using electronic cigarettes, or e-cigarettes, but the health impacts of these “safer” e-cigarette alternative have yet to be fully elucidated. E-cigarettes have been associated with inflammation and oxidative stress, which can provide selective pressures for the outgrowth of CHIP mutant cells.

Here, we evaluate the impact of e-cigarette vapor and combustible cigarette smoke on *in vitro* cell inflammatory responses and *in vivo* long-term hematopoietic differentiation. The overarching goal of this project was to understand further how an inflammatory lifestyle stressor, such as smoking, contributes to aberrant hematopoiesis in wild-type normal stem cells, Tet2-deficient cells, and JAK2^{V617F} mutant cells. In cell-based studies, cigarette smoke extract (CSE) and e-cigarette vapor extract (EVE) consistently suppress LPS-induced TNF- α secretion across macrophage/monocyte models, including primary mouse and human cells and complementary cell lines, indicating a reproducible immunosuppressive effect on mature myeloid cells. To assess consequences of smoking behavior *in vivo*, we used a custom nose-cone inhalation system to deliver controlled exposures to combustible cigarette smoke or e-cigarette aerosol to mice. Chronic exposure increased myeloid proliferation, a hallmark of HSC aging. Taken together, these results support a model in which tobacco exposures blunt innate immune responsiveness while simultaneously driving myeloid expansion conditions that accelerate hematopoietic aging and promote the expansion of mutant hematopoietic cells.

CHAPTER ONE

The intersection of hematopoiesis, inflammation, and smoking

Jeanette YC Sullivan © 2025

INTRODUCTION

Self-renewing hematopoietic stem cells (HSCs) produce billions of blood cells a day to maintain peripheral blood and immune cells in circulation. Inflammation can alter the balance of steady-state hematopoiesis, and disrupted hematopoiesis can lead to blood disorders or cancers.

Smoking is a well-known carcinogen, but the health impacts of the “safer” e-cigarette alternative have yet to be fully elucidated. This chapter will explore hematopoiesis and inflammation, the effect of both traditional and electronic cigarettes on these key processes, and the current state of research in this field.

1.1 HEMATOPOIETIC STEM CELLS

1.1.1 Introduction to hematopoiesis

Hematopoietic stem cells (HSC) were first described in the early 1900s by the German pathologist Franz Ernst Christian Neumann (1834-1918), who observed the formation of leukocytes and erythrocytes in the bone marrow and pioneered the concept of all blood cells descending from a single origin cell (Cooper 2011; Boisset and Robin 2012). Advances in structural analysis methods led to further distinction of cell types and confirmation of the self-renewing hematopoietic stem cells. As stem cells, HSCs possess the ability to self-renew, a process by which cells divide but produce at least one daughter cell that retains undifferentiated, multipotent potential. Cell proliferation is a broader term for cell division and multiplication (Fuchs and Chen 2013). This finite pool of self-renewing HSCs within our bone marrow is responsible for providing a lifetime’s worth of blood and immune cells.

Hematopoiesis occurs in waves, beginning in the yolk sac during embryonic development (Essawy et al. 2020). In “primitive hematopoiesis”, HSC precursors develop in the yolk sac, then the placenta, then the aorta-gonad-mesonephros (AGM) region (Udroiu and Sgura 2021). When the fetal liver develops, HSCs migrate there and to the spleen and thymus before taking up final primary residence in the bone marrow. A young adult male has an estimated $\sim 1.2 \times 10^{12}$ bone marrow cells and $\sim 2.8 \times 10^{13}$ blood cells (Cosgrove et al. 2021). As a research model comparison, mice have an estimated $\sim 16,000$ hematopoietic stem cells, about 0.006% of total bone marrow cells (Cosgrove et al. 2021). Once established in the bone marrow, HSCs begin the process of differentiation. Self-renewal ability and multipotent differentiation ability are the two fundamental properties of HSCs. As a cell makes differentiation decisions, it becomes increasingly committed and restricted to specific downstream paths. HSCs can be subdivided into long-term (LT-HSCs) and short-term (ST-HSCs) hematopoietic stem cells (Purton 2022). LT-HSCs maintain a mostly dormant or quiescent state. LT-HSCs differentiate into ST-HSCs, then into multipotent progenitor cells (MPP). MPPs continue to differentiate through a series of oligopotent progenitor cells, eventually committing to a myeloid or lymphoid cell fate. Lymphoid progenitor cells can become T cells, B cells, and NK cells, while myeloid progenitor cells give rise to erythrocytes (red blood cells), monocytes and macrophages, megakaryocytes and platelets, and granulocytes such as neutrophils and mast cells.

1.1.2 HSC maintenance: self-renewal and cycling

To maintain this finite pool of HSCs over a lifetime, most HSCs remain in the G0 phase of the cell cycle, which is a quiescent or dormant state (Kovtonyuk et al. 2016). When prompted, HSCs exit G0 to divide and generate daughter cells, which either preserve stemness (self-renewal

capacity) and return to quiescence or go on to further differentiation. Cell cycling is governed by cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). The dynamics of CDK binding to various phase-dependent arrangements of cyclin regulatory subunits drive cells through cell cycle transitions (Treichel and Filippi 2023). HSCs may undergo a few types of division that produce distinct outcomes. In symmetric division, a single HSC divides to produce two identical daughter cells; i.e., two self-renewal-inclined HSCs or two differentiation-inclined progenitor cells. In contrast, asymmetric division, aptly referred to as “clone splitting”, results in two daughter cells with different functions.

1.1.3 HSCs regulate innate and adaptive inflammatory immune responses

In situations such as illness, infection, or tissue damage, the body initiates the inflammatory response. Inflammation is a protective immune response that promotes healing and recruits cells to the affected site to clear the harmful stimulus. Lineage decisions are governed by environmental factors, such as inflammation, that demand increased cellular output from the bone marrow. Mature cells that egress into circulation influence the differentiation of cells still in the bone marrow. Etzrodt et al demonstrated that inflammatory cytokines directly impact key lineage-regulating transcription factors in HSCs (Etzrodt et al. 2019).

1.1.4. How “inflamm-aging” alters HSCs

Despite their remarkable capacity for self-renewal, HSCs do not have an infinite lifespan and are subject to aging, stress, and exhaustion. As the human lifespan increases, so does the importance of understanding the impact of aging on cells. Aging causes functional declines in the immune system and skewing of differentiation, often towards an increased myeloid bias (Kovtonyuk et al.

2016). The portmanteau “inflamm-aging” refers to chronic low-grade inflammation that is both caused by and contributes to aging (Mansell et al. 2023). Studies of murine HSC aging also demonstrate that aging causes a loss in clonal diversity. Sequencing by Mitchell et al estimates that HSCs accumulate ~17 mutations each year. Additionally, subjects over 75 years of age had substantially lower clonal diversity, with an average of ~15 independent clones accounting for hematopoiesis (Mitchell et al. 2022).

1.1.5 Clonal hematopoiesis: development, progression, and concerns

Over a lifetime of cell divisions, HSCs may acquire somatic mutations that provide a competitive advantage over their wild-type counterparts. The clonal expansion of a mutant HSC population is termed “clonal hematopoiesis” (CH). Clonal hematopoiesis of indeterminate potential, or CHIP, is the presence of CH without altered blood counts or a diagnosed hematologic malignancy. The incidence of CHIP increases with age and can be found in about 10% of the population by age 70 (Marston et al. 2024). Large-scale population studies have identified that the presence of these underlying CH mutations, while technically clinically benign, appears associated with a significantly increased overall risk of mortality (Moyen et al. 2025; Saadatagah et al. 2024). Clonal hematopoiesis confers a 2-4 fold increased risk of cardiovascular disease (Tajiri et al. 2023; Lu et al. 2024). CHIP has also been linked to many other inflammatory and rheumatological diseases, reinforcing the inflammatory tendencies of most CHIP mutations.

There are over 20 identified CHIP mutations, but the most common are in *JAK2*, *TET2*, *ASXL1*, and *DNMT3A*, which are also known drivers of hematological malignancies (Asada and Kitamura 2021; Xie and Zeidan 2023; Kanagal-Shamanna et al. 2024; Fabre and Vassiliou 2024; Saadatagah and Ballantyne 2023; Nathan et al. 2023). TET2, or ten-eleven translocation 2, is the

second most frequently mutated gene in CHIP (Cobo et al. 2022). TET2 is an epigenetic regulator that catalyzes the oxidation of 5-methylcytosine (5mc) to 5-hydroxymethylcytosine (5hmc) in DNA (Fuster et al. 2017; Abdel-Wahab et al. 2012). TET2 mutations have been observed in 10-20% of patients with myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN) and are associated with worse outcomes in acute myeloid leukemia (AML) (Moran-Crusio et al. 2011). *TET2*-deficient macrophages also secrete more pro-inflammatory cytokines (Jaiswal Siddhartha et al. 2017). Mutations in the Janus kinase 2 (JAK2) gene are among the most common mutations in MPN patients. The JAK2^{V617F} mutation is a conversion of valine (V) into phenylalanine (F) at the 617th amino acid of the gene. JAK2^{V617F} causes constitutive activation of JAK2. Constant activation of this JAK-STAT feedback loop can lead to increased pro-inflammatory gene expression (Geyer et al. 2015). MPN patients often have elevated levels of pro-inflammatory cytokines (Tefferi et al. 2011; Vaidya et al. 2012). CHIP mutations are inflammatory and can withstand chronic inflammation, providing a survival advantage over wild-type (WT) cells.

1.2 MYELOID CELLS

1.2.1. Monocytes

From the MPP multipotent progenitors, HSCs can progress to either common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CMPs have the ability to further mature to granulocyte-monocyte progenitors (GMPs) and subsequently monocytes, which exit the bone marrow into circulation. Monocytes are a highly heterogeneous and plastic population that play important roles in immune regulation and inflammation, both as themselves and by

differentiating into other cell types. Monocytes circulate in the blood, from which they can be drawn into tissues or recruited to sites of injury (Robinson et al. 2021).

Circulating monocytes are divided into subsets based on surface marker expression. Classical (CL) CD14⁺⁺CD16⁻ monocytes comprise ~80% of circulating monocytes, with the remaining being intermediate (IM) CD14⁺/CD16⁺ or nonclassical (NC) CD14^{low}CD16⁺⁺. For a long time, most researchers grouped the IM with the NC subset. However, more recent literature has established that the IM subset has its own distinct functional properties and gene signature (Ożańska et al. 2020). The quantity and relative frequencies of monocyte subsets are altered under pathological stressors such as infection, cancer, cardiac events, and inflammatory or autoimmune diseases (Kratofil et al. 2017). Peripheral blood monocytes can predict cardiovascular risk: in multiple studies with between 500-1000 CVD patients, elevated levels of specific monocyte subsets predicted CV death, myocardial infarction, and stroke (Hilgendorf et al. 2015).

1.2.2. Macrophages

There are many distinct macrophage populations throughout the niches of the human body. Multiple resident macrophage populations arise during embryogenesis and are derived from the yolk sac (McCabe and MacNamara 2016; Schulz et al. 2021). However, a key macrophage pipeline comes from the differentiation of monocytes from bone marrow HSCs. Macrophages, like their monocyte precursors, are also highly heterogeneous. Signaling and other environmental factors during differentiation influence the functional phenotype of a particular macrophage (Murray 2017). Key macrophage growth factors are macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 (Naito 2008). In

laboratory practices, selective combinations of growth factors are used to induce stages of polarization. Mature macrophages in the bone marrow regulate hematopoietic stem cell function (Schulz et al. 2021).

A key role of macrophages is their ability to produce and release both pro- and anti-inflammatory cytokines. Inflammatory stimuli trigger the secretion of IL-1 β , IL-18, TNF- α , IFN- γ , and more. The role of inflammasomes, such as NLRP3, has been extensively studied as regulators of inflammatory disease pathogenesis (Lévesque et al. 2021). In addition to regulating other mature immune cells via cytokine signaling, macrophages also regulate HSC quiescence and HSPC mobilization out of the bone marrow (McCabe and MacNamara 2016).

Macrophages engulf and digest pathogens, recruit other immune cells via antigen presentation, release pro- and anti-inflammatory cytokines, and more (Naito 2008; Lévesque et al. 2021; Klei et al. 2017; Heideveld and van den Akker 2017). Phagocytosis is a key mechanism of cellular engulfment that clears microorganisms, foreign substances, and apoptotic cells (Karavitis and Kovacs 2011; Ween et al. 2017). Studies have observed impaired phagocytosis checkpoints in patients with myelodysplastic syndromes (Dong et al. 2022). In atherosclerosis, monocytes infiltrate the tissue and differentiate into lesional macrophages that take up oxidized low-density lipoproteins (oxLDL) via scavenger receptors, becoming foam cells. These lipid-laden foam cells become clustered necrotic cores in atherosclerotic plaques in major arteries (Gui et al. 2022; Chen et al. 2020; Kim et al. 2020). Circulating reactive oxygen species (ROS) generate oxLDL and trigger increased expression of adhesion molecules, prompting monocyte migration and subsequent macrophage differentiation and foam cell formation (Alakhtar et al. 2023).

1.2.3. Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN) are a heterogeneous group of rare, chronic blood cancers characterized by excess proliferation of blood cells from the bone marrow. MPNs include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). PV, the most common MPN, causes an increase in red blood cells, which thickens the blood and dangerously increases the risk of thrombosis. In ET, excess platelets cause blood clots, increasing the chance of heart attacks or stroke. In PMF, the diseased bone marrow becomes inflamed and forms fibrotic scar tissue, impairing hematopoiesis. All MPNs are acquired genetic disorders, initiated by somatic mutations that arise after birth. While MPNs at their core are diseases of the blood and bone marrow, they also behave much like chronic inflammatory disorders (Soyfer and Fleischman 2023). Many of the most common MPN symptoms, such as pruritus (itching), night sweats, fatigue/weakness, rash, and fever, are also seen in inflammatory diseases. Although MPN symptoms can mostly be controlled with therapies, there is still no cure. Therefore, it is still of high clinical interest to understand the factors that promote the initiation and progression of MPNs.

Inflammation can promote MPN disease development and worsen symptoms, but its precise role is still largely undefined. Many of the foundational symptoms of MPNs are driven by cytokine overactivity. Fevers and night sweats can be exacerbated by persistent cytokine production, and patients treated with JAK inhibitors generally see clinical improvement correlated to reductions in their cytokine levels (Koschmieder et al. 2016). MPN patients have elevated levels of inflammatory cytokines, most commonly $\text{TNF}\alpha$, IL-6, and IL-1 β . MPNs are associated with inflammatory autoimmune disorders. A study of over 11,000 MPN patients found that people with a prior history of autoimmune diseases had a 20% increased risk of developing MPN, with a

2- to 3-fold risk among certain autoimmune disorders (Kristinsson et al. 2010), suggesting that chronic inflammation contributes to the genesis of MPN. Immune dysregulation undeniably impacts the development and pathogenesis of myeloproliferative neoplasms.

TNF- α is a key regulator of the delicate balance between pro- and anti-inflammatory signals and can be both myelosuppressive or myeloproliferative. TNF- α has been shown to stunt wild-type cell growth but promote leukemic cell proliferation in AML patient-derived leukemic stem cells (Volk et al. 2014) and in JAK2 V617F mutant cells (Fleischman et al. 2011). Fleischman et al. used TNF α -knockout mice to show that TNF α deficiency attenuates MPN disease progression, particularly splenomegaly (Fleischman et al. 2011). These results suggest that TNF α encourages clonal expansion and increases dominance over normal cells. In addition to TNF conferring a growth advantage to malignant cells, both malignant and normal cells contribute to aberrant cytokine production (Kleppe et al. 2015). Deranged cytokine production was observed in immature and mature cells and is modulated chiefly by STAT3 (Kleppe et al. 2015). TNF α perpetuates a self-reinforcing loop of inflammatory cytokine overproduction, creating an inflammatory environment to which the malignant clones are resistant, thereby promoting clonal expansion over wild-type cells.

Interleukin-1 β , IL-1 β , along with the related cytokine IL-1 α , binds to the IL-1 receptor 1 (IL-1R1) to regulate downstream inflammatory signaling (Rahman et al. 2022). IL-1 β is primarily produced by monocytes, macrophages, and dendritic cells (de Mooij et al. 2017). IL-1b is particularly associated with chronic myeloid leukemia, and high levels of IL-1b are linked to advanced blast phase, blast expansion, and overall poor prognosis (Arranz et al. 2017). The highest levels of IL-1b are typically found in PMF patients, and less so in those with PV or ET;

accordingly, PV patients with elevated IL-1 β are more likely to transform to fibrosis and have increased mortality (Vaidya et al. 2012). Rai et al. found that JAK2V617F mutant cells secrete IL-1 β , which then promotes the expansion of the JAK2V617F clones (Rai et al. 2023). To further delineate the importance of IL-1 β , they demonstrate that deletion of IL-1 β , deletion of the IL-1 receptor type 1 (IL-1R1), and treatment with a neutralizing IL-1 β antibody all reduce MPN disease initiation. RNA sequencing revealed that the loss of IL-1 β or IL-1R1 reduces the inflammatory gene signatures of JAK2V617F stem cells (Rai et al. 2023). These findings are analogous to those in Rahman et al., where deletion of IL-1R1 preferentially inhibited the proliferation of JAK2V617F cells in the peripheral blood of competitive bone marrow transplantation models and significantly reduced bone marrow fibrosis (Rahman et al. 2022). Flores-Gomez et al. demonstrate that exposing CD34⁺ HSPCs to IL-1 β increased myeloid cell proliferation and overall absolute colony counts in a colony-forming unit (CFU) assay (Flores-Gomez et al. 2024). Although IL-1 β did not alter monocyte differentiation or their subset distribution, exposure to IL-1 β increased LPS-induced IL-1 β and TNF α secretion (Flores-Gomez et al. 2024).

Neutralizing IL-1 β signaling is a popular target for treating inflammatory conditions.

Canakinumab, a fully human monoclonal antibody targeting IL-1 β , was first approved in 2009 for a rare type of cryopyrin-associated autoinflammatory syndrome and has since been sparingly approved for other inflammatory disorders. The CANTOS trial demonstrated that canakinumab treatment reduced the overall incidence of cardiovascular events, which may be of relevance to MPN patients at risk of stroke or thrombosis. Altogether, the literature demonstrates that IL-1 β is a key regulator of JAK2V617F cells across all stages of the disorder, from initiating a few CHIP clones into MPN to driving inflammation and fibrosis in chronic, established MPNs. A major

inducer of IL-1 β and other inflammatory cytokines is the NF- κ B signaling pathway, whose role is intertwined with the NLRP3 (Nod-like receptor protein 3) inflammasome. NLRP3 is a cytosolic receptor expressed on hematopoietic stem and progenitor cells (HSPCs) and peripheral blood cells (Ratajczak et al. 2020). The NLRP3 protein converts pro-IL-1 β and pro-IL-18 into their active forms (Parciante et al. 2023). The NF- κ B transcription factor mediates NLRP3 inflammasome assembly and may be activated by cytokines, lipopolysaccharide (LPS), or other stimuli. NLRP3 inflammasome activity has been more extensively characterized in myelodysplastic disorders (MDS), but, as the key facilitator of cleavage of IL-1 β and IL-18, its role and potential therapeutic target in MPN warrant examination.

IL-6 is elevated in MPN patients and murine models. Interleukin-6 plays a key role in many physiological processes, including inflammation, hematopoiesis, bone metabolism, and more. It is a target of the NF- κ B inflammasome. Fibroblast production of IL-6 may contribute to bone marrow fibrosis. A Mendelian randomization study of over 100,000 people, using genotyping, demonstrated that a loss-of-function polymorphism in IL6R, the IL-6 receptor gene, reduced the risk of JAK2V617F mutations and MPN diagnosis (Pedersen et al. 2020). However, Baldauf et al. found that anti-IL-6 antibody treatment did not improve splenomegaly, hematocrit, and other disease hallmarks in a polycythemia vera mouse model (Baldauf et al. 2022).

1.3. TRADITIONAL AND ELECTRONIC CIGARETTES

1.3.1. Combustible tobacco cigarettes

Tobacco use is the leading contributor to preventable illness and death, accounting for half a million deaths in the United States each year. Classic cigarettes contain a mix of tobacco leaves

(and fillers) that release inhalable nicotine as they burn. Over 7000 chemicals have been identified in traditional cigarette smoke, including almost a hundred carcinogenic chemicals (Zarcone et al. 2023). Cigarettes include heavy metals, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), and more carcinogenic substances. Smoking increases the risk of cancers, chronic obstructive pulmonary diseases, cardiovascular and neurodegenerative diseases, and more. In the lungs, cigarette smoking has been observed to increase recruitment of macrophages and neutrophils, but the influx of cells is less effective (Gualano et al. 2008).

1.3.2. Electronic cigarettes

Electronic cigarettes, or e-cigarettes have been promoted as a smoking alternative/cessation device. E-cigarettes are battery-operated devices that vaporize the e-liquid, which typically consists of a 60/40 or 70/30 mix of propylene glycol and vegetable glycerin with added flavorings and nicotine. The logic is that “heat not smoke” is a less harmful way to consume nicotine. E-cigarettes were first invented in China in 2003 and hit the US markets in 2007, with the JUUL brand being the most common. Cases of E-cigarette vaping-associated acute lung injury (EVALI) were sensationalized, but most of these cases were attributed to the presence of vitamin E acetate in counterfeit e-cigarettes (Marrocco et al. 2022). However, since then, new brands, flavors, and generations of e-cigarettes have emerged, and there are still millions of young consumers using e-cigarettes. These inventions are a double-edged sword because while they have helped smokers quit, e-cigarettes have also become popular with non-cigarette smokers and young tweens and teenagers. In the US alone there are 10 million adults and over 5

million middle/high school students using e-cigarettes (Mirbolouk et al. 2018). (Zima et al. 2025)

1.3.3. Cigarettes on inflammatory cytokine production

Literature on the impact of cigarette smoke, e-cigarettes, their extracts, and/or nicotine alone on myeloid cells is highly contradictory. A survey of studies looking at the impact of cigarette or e-cigarette components on pro- and anti-inflammatory cytokines production gives conflicting results. In support of cigarette smoke extract (CSE) or e-cigarette vape extract (EVE) being immunosuppressive, Ouyang et al. showed that CSE decreased IL-1b, TNF, IL-2, and IFN production by PBMCs, and nicotine-only treatment also decreased TNF- α (Ouyang et al. 2000). Similarly, Arimilli et al. found that CSE decreased TNF, IL-8, and IL-6 production in PBMCs, a defect that could be rescued by NAC (Arimilli et al. 2017). Ween et al. observed that CSE, EVE, and nicotine decreased TNF, IL-6, and IL-1 β in THP-1 macrophages, and Hamon et al. describe decreased inflammatory cytokine production in human monocyte-derived macrophages after CSE and EVE exposure (Ween et al. 2017; Hamon et al. 2024). Chen et al. saw inhibited expression of TNF- α , IL-1 β , and IL-6 in LPS-stimulated macrophages from smokers compared to non-smokers (Chen et al. 2007). Breivik et al demonstrated that nicotine-treated rats had inhibited levels of LPS-induced TNF- α , IL-1b, and IL-10 (Breivik et al. 2009).

In contrast, many studies have also observed increased pro-inflammatory cytokine production and indications of overall pro-inflammatory effects by combustible or electronic cigarettes. Zhao et al found that both tobacco cigarette and electronic cigarette aerosols increased mRNA expression and protein secretion of TNF- α , IL-6, and IL-1 β in RAW264.7 macrophages (Zhao et al. 2023). Mehta and Dhawan saw increased IL-1 and IL-18 by cigarette smoke extract in THP-1 monocytes, THP-1 macrophages, and THP-1 foam cells (Mehta and Dhawan 2020). Giebe et al

saw that cigarette and e-cigarette extracts, but not nicotine alone, increased TNF- α in THP-1 monocytes (Giebe et al. 2023). Clearly, the sum impact of these products is complex and differs greatly between environments.

1.3.5. Cigarettes and clonal hematopoiesis

Cigarette smoke is also strongly associated with the development of CHIP (Levin et al. 2022; Dawoud et al. 2020; Kessler et al. 2022; Lee et al. 2024). Analysis of the UK Biobank (UKB) cohort (n=502,524) identified a significant association ($p < 0.0005$) between smoking behavior and incidence of CHIP (Levin et al. 2022). A separate UKB analysis confirmed this link, showing that myeloid clonal hematopoiesis was particularly enriched among current smokers compared with past smokers (Dawoud et al. 2020). Similarly, exome sequencing from the Geisinger MyCode Community Health Initiative revealed that heavy smoking was strongly associated with CHIP carrier status (Kessler et al. 2022). Importantly, across both the UKB and Geisinger cohorts, CHIP carriers showed an increased risk of hematologic cancers, particularly myeloproliferative neoplasms (Kessler et al. 2022). Evidence is also mounting that other inhaled environmental exposures can impact the development of CHIP. For example, World Trade Center (WTC)-exposed first responders had significantly more CHIP ($p = 0.0006$) compared to non-WTC-exposed firefighters, with the most mutations in *DNMT3A* and *TET2* (Jasra et al. 2022). Altogether, evidence suggests that inhaled toxins, particularly cigarette smoking, can substantially alter hematopoiesis and provide an advantageous environment for the proliferation of mutant HSC clones.

Smoking is theorized to induce a chronic low-grade inflammatory physiological state. Chronic inflammation is proposed to drive clonal evolution in MPNs (Hasselbalch 2012), and further

studies have reinforced a link between smoking and the development of polycythemia vera (PV) (Hasselbalch 2015). Pedersen et al identified a significant association between smoking and MPN development in the Danish Health Examination Survey cohort (Pedersen et al. 2018). Smoking can also alter the bone marrow niche, leading to HSC dysfunction (Siggins et al. 2014).

CONCLUSIONS: BRIDGING TOXICOLOGY AND HEMATOPOIESIS

Environmental exposures have significant impacts on human health and disease. Smoking is a prominent public health issue. As the average age of the population increases, research into the impacts of aging, particularly on hematopoiesis, becomes increasingly relevant. Here, we leverage the common associations between smoking and clonal hematopoiesis to understand how they impact hematopoiesis and immune responses. In theory, many of the carcinogenic impacts of smoking stem from the hazardous chemicals in tobacco cigarettes that are linked to oxidative stress and DNA damage. This leads to the conception that e-cigarette alternatives are safer. However, comparative studies of e-cigarettes are limited and contradictory. Several studies have identified correlations between smoking and the development of clonal hematopoiesis, but the mechanisms behind this association remain largely unknown.

REFERENCES

- Abdel-Wahab, Omar, Ayalew Tefferi, and Ross L. Levine. 2012. "Role of TET2 and ASXL1 Mutations in the Pathogenesis of Myeloproliferative Neoplasms." *Hematology/Oncology Clinics of North America* 26 (5): 1053–64. <https://doi.org/10.1016/j.hoc.2012.07.006>.
- Alakhtar, Bayan, Cynthia Guilbert, Nivetha Subramaniam, et al. 2023. "E-Cigarette Exposure Causes Early pro-Atherogenic Changes in an Inducible Murine Model of Atherosclerosis." *Frontiers in Toxicology* 5 (December): 1244596. <https://doi.org/10.3389/ftox.2023.1244596>.
- Arimilli, Subhashini, Eckhardt Schmidt, Brad E. Damratoski, and G. L. Prasad. 2017. "Role of Oxidative Stress in the Suppression of Immune Responses in Peripheral Blood Mononuclear Cells Exposed to Combustible Tobacco Product Preparation." *Inflammation* 40 (5): 1622–30. <https://doi.org/10.1007/s10753-017-0602-9>.
- Arranz, Lorena, Maria del Mar Arriero, and Alicia Villatoro. 2017. "Interleukin-1 β as Emerging Therapeutic Target in Hematological Malignancies and Potentially in Their Complications." *Blood Reviews* 31 (5): 306–17. <https://doi.org/10.1016/j.blre.2017.05.001>.
- Asada, Shuhei, and Toshio Kitamura. 2021. "Clonal Hematopoiesis and Associated Diseases: A Review of Recent Findings." *Cancer Science* 112 (10): 3962–71. <https://doi.org/10.1111/cas.15094>.
- Baldauf, Conny K., Peter Müller, Tobias R. Haage, et al. 2022. "Anti-IL-6 Cytokine Treatment Has No Impact on Elevated Hematocrit or Splenomegaly in a Polycythemia Vera Mouse Model." *Blood Advances* 6 (2): 399–404. <https://doi.org/10.1182/bloodadvances.2021004379>.
- Boisset, Jean-Charles, and Catherine Robin. 2012. "On the Origin of Hematopoietic Stem Cells: Progress and Controversy." *Stem Cell Research* 8 (1): 1–13. <https://doi.org/10.1016/j.scr.2011.07.002>.
- Breivik, T., Y. Gundersen, P. Gjermo, S. Von Hörsten, and P. K. Opstad. 2009. "Nicotinic Acetylcholine Receptor Activation Mediates Nicotine-Induced Enhancement of Experimental Periodontitis." *Journal of Periodontal Research* 44 (3): 297–304. <https://doi.org/10.1111/j.1600-0765.2009.01223.x>.
- Chen, Haiyan, Mark J. Cowan, Jeffrey D. Hasday, Stefanie N. Vogel, and Andrei E. Medvedev. 2007. "Tobacco Smoking Inhibits Expression of Proinflammatory Cytokines and Activation of IL-1R-Associated Kinase, P38, and NF-kappaB in Alveolar Macrophages Stimulated with TLR2 and TLR4 Agonists." *Journal of Immunology (Baltimore, Md.: 1950)* 179 (9): 6097–106. <https://doi.org/10.4049/jimmunol.179.9.6097>.
- Chen, Wujun, Yudong Wu, Qi Lu, Shuai Wang, and Dongming Xing. 2020. "Endogenous ApoA-I Expression in Macrophages: A Potential Target for Protection against Atherosclerosis." *Clinica Chimica Acta* 505 (June): 55–59. <https://doi.org/10.1016/j.cca.2020.02.025>.
- Cobo, Isidoro, Tiffany Tanaka, Christopher K. Glass, and Calvin Yeang. 2022. "Clonal Hematopoiesis Driven by DNMT3A and TET2 Mutations: Role in Monocyte and Macrophage Biology and Atherosclerotic Cardiovascular Disease." *Current Opinion in Hematology* 29 (1): 1–7. <https://doi.org/10.1097/MOH.0000000000000688>.

- Cooper, Barry. 2011. "The Origins of Bone Marrow as the Seedbed of Our Blood: From Antiquity to the Time of Osler." *Proceedings (Baylor University. Medical Center)* 24 (2): 115–18. <https://doi.org/10.1080/08998280.2011.11928697>.
- Cosgrove, Jason, Lucie S. P. Hustin, Rob J. de Boer, and Leïla Perié. 2021. "Hematopoiesis in Numbers." *Trends in Immunology* 42 (12): 1100–1112. <https://doi.org/10.1016/j.it.2021.10.006>.
- Dawoud, Ahmed A. Z., William J. Tapper, and Nicholas C. P. Cross. 2020. "Clonal Myelopoiesis in the UK Biobank Cohort: ASXL1 Mutations Are Strongly Associated with Smoking." *Leukemia* 34 (10): 10. <https://doi.org/10.1038/s41375-020-0896-8>.
- Dong, Xifeng, Yu Han, Yumei Liu, et al. 2022. "Phagocytosis Checkpoints on Hematopoietic Stem Cells in Patients with Myelodysplastic Syndromes." *Asia-Pacific Journal of Clinical Oncology* 18 (2): e119–28. <https://doi.org/10.1111/ajco.13566>.
- Essawy, Mohamed, Ahmed Abdelfattah-Hassan, Eman Radwan, Mostafa F. Abdelhai, S. Elshaboury, and Nagwa El-Badri. 2020. "Hematopoietic Stem Cells and Control of Hematopoiesis." In *Regenerative Medicine and Stem Cell Biology*, edited by Nagwa El-Badri. Springer International Publishing. https://doi.org/10.1007/978-3-030-55359-3_3.
- Etzrodt, Martin, Nouraz Ahmed, Philipp S. Hoppe, et al. 2019. "Inflammatory Signals Directly Instruct PU.1 in HSCs via TNF." *Blood* 133 (8): 816–19. <https://doi.org/10.1182/blood-2018-02-832998>.
- Fabre, Margarete A., and George S. Vassiliou. 2024. "The Lifelong Natural History of Clonal Hematopoiesis and Its Links to Myeloid Neoplasia." *Blood* 143 (7): 573–81. <https://doi.org/10.1182/blood.2023019964>.
- Fleischman, Angela G., Karl J. Aichberger, Samuel B. Luty, et al. 2011. "TNF α Facilitates Clonal Expansion of JAK2V617F Positive Cells in Myeloproliferative Neoplasms." *Blood* 118 (24): 6392–98. <https://doi.org/10.1182/blood-2011-04-348144>.
- Flores-Gomez, Daniela, Willemijn Hobo, Diede van Ens, et al. 2024. "Interleukin-1 β Induces Trained Innate Immunity in Human Hematopoietic Progenitor Cells *in Vitro*." *Stem Cell Reports* 19 (12): 1651–64. <https://doi.org/10.1016/j.stemcr.2024.09.004>.
- Fuchs, Elaine, and Ting Chen. 2013. "A Matter of Life and Death: Self-Renewal in Stem Cells." *EMBO Reports* 14 (1): 39–48. <https://doi.org/10.1038/embor.2012.197>.
- Fuster, José J., Susan MacLauchlan, María A. Zuriaga, et al. 2017. "Clonal Hematopoiesis Associated with TET2 Deficiency Accelerates Atherosclerosis Development in Mice." *Science* 355 (6327): 842–47. <https://doi.org/10.1126/science.aag1381>.
- Geyer, Holly L., Amylou C. Dueck, Robyn M. Scherber, and Ruben A. Mesa. 2015. "Impact of Inflammation on Myeloproliferative Neoplasm Symptom Development." *Mediators of Inflammation* 2015 (1): 284706. <https://doi.org/10.1155/2015/284706>.
- Giebe, Sindy, Melanie Brux, Anja Hofmann, et al. 2023. "Comparative Study of the Effects of Cigarette Smoke versus Next-Generation Tobacco and Nicotine Product Extracts on Inflammatory Biomarkers of Human Monocytes." *Pflügers Archiv - European Journal of Physiology* 475 (7): 823–33. <https://doi.org/10.1007/s00424-023-02809-9>.
- Gualano, Rosa C., Michelle J. Hansen, Ross Vlahos, et al. 2008. "Cigarette Smoke Worsens Lung Inflammation and Impairs Resolution of Influenza Infection in Mice." *Respiratory Research* 9 (1): 53. <https://doi.org/10.1186/1465-9921-9-53>.
- Gui, Yuzhou, Hongchao Zheng, and Richard Y. Cao. 2022. "Foam Cells in Atherosclerosis: Novel Insights Into Its Origins, Consequences, and Molecular Mechanisms." *Frontiers in Cardiovascular Medicine* 9 (April). <https://doi.org/10.3389/fcvm.2022.845942>.

- Hamon, Rhys, Leigh Thredgold, Asiri Wijenayaka, Nicole Anne Bastian, and Miranda P. Ween. 2024. "Dual Exposure to E-Cigarette Vapour and Cigarette Smoke Results in Poorer Airway Cell, Monocyte, and Macrophage Function Than Single Exposure." *International Journal of Molecular Sciences* 25 (11): 11. <https://doi.org/10.3390/ijms25116071>.
- Hasselbalch, Hans Carl. 2012. "Perspectives on Chronic Inflammation in Essential Thrombocythemia, Polycythemia Vera, and Myelofibrosis: Is Chronic Inflammation a Trigger and Driver of Clonal Evolution and Development of Accelerated Atherosclerosis and Second Cancer?" *Blood* 119 (14): 3219–25. <https://doi.org/10.1182/blood-2011-11-394775>.
- Hasselbalch, Hans Carl. 2015. "Smoking as a Contributing Factor for Development of Polycythemia Vera and Related Neoplasms." *Leukemia Research* 39 (11): 1137–45. <https://doi.org/10.1016/j.leukres.2015.09.002>.
- Heideveld, Esther, and Emile van den Akker. 2017. "Digesting the Role of Bone Marrow Macrophages on Hematopoiesis." *Immunobiology*, European Macrophage and Dendritic Cell Society (EMDS) Special Issue: Monocytes, dendritic cells and macrophages - basic research and pathophysiological aspects, vol. 222 (6): 814–22. <https://doi.org/10.1016/j.imbio.2016.11.007>.
- Hilgendorf, Ingo, Filip K. Swirski, and Clinton S. Robbins. 2015. "Monocyte Fate in Atherosclerosis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 35 (2): 272–79. <https://doi.org/10.1161/ATVBAHA.114.303565>.
- Jaiswal Siddhartha, Natarajan Pradeep, Silver Alexander J., et al. 2017. "Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease." *New England Journal of Medicine* 377 (2): 111–21. <https://doi.org/10.1056/NEJMoa1701719>.
- Jasra, Sakshi, Orsi Giricz, Rachel Zeig-Owens, et al. 2022. "High Burden of Clonal Hematopoiesis in First Responders Exposed to the World Trade Center Disaster." *Nature Medicine* 28 (3): 468–71. <https://doi.org/10.1038/s41591-022-01708-3>.
- Kanagal-Shamanna, Rashmi, David B. Beck, and Katherine R. Calvo. 2024. "Clonal Hematopoiesis, Inflammation, and Hematologic Malignancy." *Annual Review of Pathology: Mechanisms of Disease* 19 (Volume 19, 2024): 479–506. <https://doi.org/10.1146/annurev-pathmechdis-051222-122724>.
- Karavitis, John, and Elizabeth J Kovacs. 2011. "Macrophage Phagocytosis: Effects of Environmental Pollutants, Alcohol, Cigarette Smoke, and Other External Factors." *Journal of Leukocyte Biology* 90 (6): 1065–78. <https://doi.org/10.1189/jlb.0311114>.
- Kessler, Michael D., Amy Damask, Sean O’Keeffe, et al. 2022. "Common and Rare Variant Associations with Clonal Haematopoiesis Phenotypes." *Nature* 612 (7939): 301–9. <https://doi.org/10.1038/s41586-022-05448-9>.
- Kim, Ki-Wook, Stoyan Ivanov, and Jesse W. Williams. 2020. "Monocyte Recruitment, Specification, and Function in Atherosclerosis." *Cells* 10 (1): 15. <https://doi.org/10.3390/cells10010015>.
- Klei, Thomas R. L., Sanne M. Meinderts, Timo K. van den Berg, and Robin van Bruggen. 2017. "From the Cradle to the Grave: The Role of Macrophages in Erythropoiesis and Erythrophagocytosis." *Frontiers in Immunology* 8 (February). <https://doi.org/10.3389/fimmu.2017.00073>.
- Kleppe, Maria, Minsuk Kwak, Priya Koppikar, et al. 2015. "JAK–STAT Pathway Activation in Malignant and Nonmalignant Cells Contributes to MPN Pathogenesis and Therapeutic

- Response.” *Cancer Discovery* 5 (3): 316–31. <https://doi.org/10.1158/2159-8290.CD-14-0736>.
- Koschmieder, S., T. I. Mughal, H. C. Hasselbalch, et al. 2016. “Myeloproliferative Neoplasms and Inflammation: Whether to Target the Malignant Clone or the Inflammatory Process or Both.” *Leukemia* 30 (5): 5. <https://doi.org/10.1038/leu.2016.12>.
- Kovtonyuk, Larisa V., Kristin Fritsch, Xiaomin Feng, Markus G. Manz, and Hitoshi Takizawa. 2016. “Inflamm-Aging of Hematopoiesis, Hematopoietic Stem Cells, and the Bone Marrow Microenvironment.” *Frontiers in Immunology* 7 (November). <https://doi.org/10.3389/fimmu.2016.00502>.
- Kratofil, Rachel M., Paul Kubes, and Justin F. Deniset. 2017. “Monocyte Conversion During Inflammation and Injury.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 37 (1): 35–42. <https://doi.org/10.1161/ATVBAHA.116.308198>.
- Kristinsson, Sigurdur Y., Ola Landgren, Jan Samuelsson, Magnus Björkholm, and Lynn R. Goldin. 2010. “Autoimmunity and the Risk of Myeloproliferative Neoplasms.” *Haematologica* 95 (7): 1216–20. <https://doi.org/10.3324/haematol.2009.020412>.
- Lee, Jung-Kyu, Hongyul An, Youngil Koh, and Chang-Hoon Lee. 2024. “Clonal Hematopoiesis of Indeterminate Potential Is Associated with Current Smoking Status and History of Exacerbation in Patients with Chronic Obstructive Pulmonary Disease.” *Tuberculosis and Respiratory Diseases*, ahead of print, February 6. <https://doi.org/10.4046/trd.2023.0165>.
- Lévesque, Jean-Pierre, Kim M. Summers, Susan M. Millard, Kavita Bisht, Ingrid G. Winkler, and Allison R. Pettit. 2021. “Role of Macrophages and Phagocytes in Orchestrating Normal and Pathologic Hematopoietic Niches.” *Experimental Hematology* 100 (August): 12–31.e1. <https://doi.org/10.1016/j.exphem.2021.07.001>.
- Levin, Michael G., Tetsushi Nakao, Seyedeh M. Zekavat, et al. 2022. “Genetics of Smoking and Risk of Clonal Hematopoiesis.” *Scientific Reports* 12 (1): 7248. <https://doi.org/10.1038/s41598-022-09604-z>.
- Lu, Lijun, Wangwei Jing, Weiming Qian, Lin Fan, and Jifang Cheng. 2024. “Association between Dietary Patterns and Cardiovascular Diseases: A Review.” *Current Problems in Cardiology* 49 (3): 102412. <https://doi.org/10.1016/j.cpcardiol.2024.102412>.
- Mansell, Els, Dawn S. Lin, Stephen J. Loughran, Michael D. Milsom, and Jennifer J. Trowbridge. 2023. “New Insight into the Causes, Consequences, and Correction of Hematopoietic Stem Cell Aging.” *Experimental Hematology* 125–126 (September): 1–5. <https://doi.org/10.1016/j.exphem.2023.07.002>.
- Marrocco, Antonella, Dilpreet Singh, David C. Christiani, and Philip Demokritou. 2022. “E-Cigarette Vaping Associated Acute Lung Injury (EVALI): State of Science and Future Research Needs.” *Critical Reviews in Toxicology* 52 (3): 188–220. <https://doi.org/10.1080/10408444.2022.2082918>.
- Marston, Nicholas A., James P. Pirruccello, Giorgio E. M. Melloni, et al. 2024. “Clonal Hematopoiesis, Cardiovascular Events and Treatment Benefit in 63,700 Individuals from Five TIMI Randomized Trials.” *Nature Medicine*, August 6, 1–7. <https://doi.org/10.1038/s41591-024-03188-z>.
- McCabe, Amanda, and Katherine C. MacNamara. 2016. “Macrophages: Key Regulators of Steady-State and Demand-Adapted Hematopoiesis.” *Experimental Hematology* 44 (4): 213–22. <https://doi.org/10.1016/j.exphem.2016.01.003>.
- Mehta, Sakshi, and Veena Dhawan. 2020. “Exposure of Cigarette Smoke Condensate Activates NLRP3 Inflammasome in THP-1 Cells in a Stage-Specific Manner: An Underlying Role

- of Innate Immunity in Atherosclerosis.” *Cellular Signalling* 72 (August): 109645. <https://doi.org/10.1016/j.cellsig.2020.109645>.
- Mirbolouk, Mohammadhassan, Paniz Charkhchi, Sina Kianoush, et al. 2018. “Prevalence and Distribution of E-Cigarette Use Among U.S. Adults: Behavioral Risk Factor Surveillance System, 2016.” *Annals of Internal Medicine* 169 (7): 429–38. <https://doi.org/10.7326/M17-3440>.
- Mitchell, Emily, Michael Spencer Chapman, Nicholas Williams, et al. 2022. “Clonal Dynamics of Haematopoiesis across the Human Lifespan.” *Nature* 606 (7913): 343–50. <https://doi.org/10.1038/s41586-022-04786-y>.
- Mooij, Charlotte E. M. de, Mihai G. Netea, Walter J. F. M. van der Velden, and Nicole M. A. Blijlevens. 2017. “Targeting the Interleukin-1 Pathway in Patients with Hematological Disorders.” *Blood* 129 (24): 3155–64. <https://doi.org/10.1182/blood-2016-12-754994>.
- Moran-Crusio, Kelly, Linsey Reavie, Alan Shih, et al. 2011. “Tet2 Loss Leads to Increased Hematopoietic Stem Cell Self-Renewal and Myeloid Transformation.” *Cancer Cell* 20 (1): 11–24. <https://doi.org/10.1016/j.ccr.2011.06.001>.
- Moyen, Thaïs B., Victoria Tomaz, Luiz Gustavo F. Cortes, et al. 2025. “Prospective Characterization of Clonal Hematopoiesis and Its Clinical Associations in Elderly Patients.” *Scientific Reports* 15 (1): 40824. <https://doi.org/10.1038/s41598-025-24475-w>.
- Murray, Peter J. 2017. “Macrophage Polarization.” *Annual Review of Physiology* 79 (Volume 79, 2017): 541–66. <https://doi.org/10.1146/annurev-physiol-022516-034339>.
- Naito, Makoto. 2008. “Macrophage Differentiation and Function in Health and Disease.” *Pathology International* 58 (3): 143–55. <https://doi.org/10.1111/j.1440-1827.2007.02203.x>.
- Nathan, Daniel I., Max Dougherty, Manasa Bhatta, John Mascarenhas, and Bridget K. Marcellino. 2023. “Clonal Hematopoiesis and Inflammation: A Review of Mechanisms and Clinical Implications.” *Critical Reviews in Oncology/Hematology* 192 (December): 104187. <https://doi.org/10.1016/j.critrevonc.2023.104187>.
- Ouyang, Yanli, Nisha Virasch, Ping Hao, et al. 2000. “Suppression of Human IL-1 β , IL-2, IFN- γ , and TNF- α Production by Cigarette Smoke Extracts.” *Journal of Allergy and Clinical Immunology* 106 (2): 280–87. <https://doi.org/10.1067/mai.2000.107751>.
- Ożańska, Agnieszka, Donata Szymczak, and Justyna Rybka. 2020. “Pattern of Human Monocyte Subpopulations in Health and Disease.” *Scandinavian Journal of Immunology* 92 (1): e12883. <https://doi.org/10.1111/sji.12883>.
- Parciante, Elisa, Cosimo Cumbo, Luisa Anelli, et al. 2023. “The Role of NLRP3, a Star of Excellence in Myeloproliferative Neoplasms.” *International Journal of Molecular Sciences* 24 (5): 5. <https://doi.org/10.3390/ijms24054860>.
- Pedersen, Kasper M., Marie Bak, Anders L. Sørensen, et al. 2018. “Smoking Is Associated with Increased Risk of Myeloproliferative Neoplasms: A General Population-Based Cohort Study.” *Cancer Medicine* 7 (11): 5796–802. <https://doi.org/10.1002/cam4.1815>.
- Pedersen, Kasper Mønsted, Yunus Çolak, Christina Ellervik, Hans Carl Hasselbalch, Stig Egil Bojesen, and Børge Grønne Nordestgaard. 2020. “Loss-of-Function Polymorphism in IL6R Reduces Risk of JAK2V617F Somatic Mutation and Myeloproliferative Neoplasm: A Mendelian Randomization Study.” *eClinicalMedicine* 21 (April). <https://doi.org/10.1016/j.eclinm.2020.100280>.

- Purton, Louise E. 2022. "Adult Murine Hematopoietic Stem Cells and Progenitors: An Update on Their Identities, Functions, and Assays." *Experimental Hematology* 116 (December): 1–14. <https://doi.org/10.1016/j.exphem.2022.10.005>.
- Rahman, Mohammed Ferdous-Ur, Yue Yang, Bao T. Le, et al. 2022. "Interleukin-1 Contributes to Clonal Expansion and Progression of Bone Marrow Fibrosis in JAK2V617F-Induced Myeloproliferative Neoplasm." *Nature Communications* 13 (1): 1. <https://doi.org/10.1038/s41467-022-32928-3>.
- Rai, Shivam, Yang Zhang, Elodie Grockowiak, et al. 2023. *IL-1 β Promotes MPN Disease Initiation by Favoring Early Clonal Expansion of JAK2 -Mutant Hematopoietic Stem Cells*. Preprint. Cancer Biology. <https://doi.org/10.1101/2023.05.21.541610>.
- Ratajczak, Mariusz Z., Kamila Bujko, Monika Cymer, et al. 2020. "The Nlrp3 Inflammasome as a 'Rising Star' in Studies of Normal and Malignant Hematopoiesis." *Leukemia* 34 (6): 6. <https://doi.org/10.1038/s41375-020-0827-8>.
- Robinson, Amy, Claudia Z. Han, Christopher K. Glass, and Jeffrey W. Pollard. 2021. "Monocyte Regulation in Homeostasis and Malignancy." *Trends in Immunology* 42 (2): 104–19. <https://doi.org/10.1016/j.it.2020.12.001>.
- Saadatagah, Seyedmohammad, and Christie M. Ballantyne. 2023. "Clonal Hematopoiesis of Indeterminate Potential and Cardiovascular Disease." *Translational Research* 255 (May): 152–58. <https://doi.org/10.1016/j.trsl.2022.08.013>.
- Saadatagah, Seyedmohammad, Md Mesbah Uddin, Lachelle D. Weeks, et al. 2024. "Clonal Hematopoiesis Risk Score and All-Cause and Cardiovascular Mortality in Older Adults." *JAMA Network Open* 7 (1): e2351927. <https://doi.org/10.1001/jamanetworkopen.2023.51927>.
- Schulz, Christian, Tobias Petzold, and Hellen Ishikawa-Ankerhold. 2021. "Macrophage Regulation of Granulopoiesis and Neutrophil Functions." *Antioxidants & Redox Signaling* 35 (3): 182–91. <https://doi.org/10.1089/ars.2020.8203>.
- Siggins, Robert W., Fokhrul Hossain, Tayyab Rehman, John N. Melvan, Ping Zhang, and David A. Welsh. 2014. "Cigarette Smoke Alters the Hematopoietic Stem Cell Niche." *Medical Sciences* 2 (1): 37–50. <https://doi.org/10.3390/medsci2010037>.
- Soyfer, Eli M., and Angela G. Fleischman. 2023. "Myeloproliferative Neoplasms – Blurring the Lines between Cancer and Chronic Inflammatory Disorder." *Frontiers in Oncology* 13 (June). <https://doi.org/10.3389/fonc.2023.1208089>.
- Tajiri, Kazuko, Yasuhito Suehara, Toshio Suzuki, and Ikuo Sekine. 2023. "Clonal Hematopoiesis and Associated Cardiovascular Diseases." *Japanese Journal of Clinical Oncology* 53 (3): 187–94. <https://doi.org/10.1093/jjco/hyac210>.
- Tefferi, Ayalew, Rakhee Vaidya, Domenica Caramazza, Christy Finke, Terra Lasho, and Animesh Pardanani. 2011. "Circulating Interleukin (IL)-8, IL-2R, IL-12, and IL-15 Levels Are Independently Prognostic in Primary Myelofibrosis: A Comprehensive Cytokine Profiling Study." *Journal of Clinical Oncology* 29 (10): 1356–63. <https://doi.org/10.1200/JCO.2010.32.9490>.
- Treichel, Sydney, and Marie-Dominique Filippi. 2023. "Linking Cell Cycle to Hematopoietic Stem Cell Fate Decisions." *Frontiers in Cell and Developmental Biology* 11 (August): 1231735. <https://doi.org/10.3389/fcell.2023.1231735>.
- Udroiu, Ion, and Antonella Sgura. 2021. "Growing and Aging of Hematopoietic Stem Cells." *World Journal of Stem Cells* 13 (6): 594–604. <https://doi.org/10.4252/wjsc.v13.i6.594>.

- Vaidya, Rakhee, Naseema Gangat, Thitina Jimma, et al. 2012. "Plasma Cytokines in Polycythemia Vera: Phenotypic Correlates, Prognostic Relevance, and Comparison with Myelofibrosis." *American Journal of Hematology* 87 (11): 1003–5. <https://doi.org/10.1002/ajh.23295>.
- Volk, Andrew, Jing Li, Junping Xin, et al. 2014. "Co-Inhibition of NF- κ B and JNK Is Synergistic in TNF-Expressing Human AML." *Journal of Experimental Medicine* 211 (6): 1093–108. <https://doi.org/10.1084/jem.20130990>.
- Ween, Miranda P., Jonathan J. Whittall, Rhys Hamon, Paul N. Reynolds, and Sandra J. Hodge. 2017. "Phagocytosis and Inflammation: Exploring the Effects of the Components of E-Cigarette Vapor on Macrophages." *Physiological Reports* 5 (16): e13370. <https://doi.org/10.14814/phy2.13370>.
- Xie, Zhuoer, and Amer M. Zeidan. 2023. "CHIPing Away the Progression Potential of CHIP: A New Reality in the Making." *Blood Reviews* 58 (March): 101001. <https://doi.org/10.1016/j.blre.2022.101001>.
- Zarcone, Gianni, Marie Lenski, Thomas Martinez, et al. 2023. "Impact of Electronic Cigarettes, Heated Tobacco Products and Conventional Cigarettes on the Generation of Oxidative Stress and Genetic and Epigenetic Lesions in Human Bronchial Epithelial BEAS-2B Cells." *Toxics* 11 (10): 10. <https://doi.org/10.3390/toxics11100847>.
- Zhao, Minghan, Yuting Han, Qi Yang, et al. 2023. "Evaluation of the Effects of E-Cigarette Aerosol Extracts and Tobacco Cigarette Smoke Extracts on RAW264.7 Cells." *ACS Omega* 8 (32): 29336–45. <https://doi.org/10.1021/acsomega.3c02758>.
- Zima, Katarzyna, Aleksandra Bogucka, Miłosz Wojtas, and Magdalena Zabielska-Kaczorowska. 2025. "Immunological Effects of Electronic Cigarette Use: A Review of Current Evidence." *Clinical Reviews in Allergy & Immunology* 68 (1): 9. <https://doi.org/10.1007/s12016-025-09026-5>.

CHAPTER TWO

Cigarette Smoke and E-Cigarette Aerosol Extracts Induce Myelopoiesis and Suppress Inflammatory Cytokine Production

This chapter is from a manuscript that has been submitted for publication.

Jeanette YC Sullivan © 2025

Cigarette Smoke and E-Cigarette Aerosol Extracts Induce Myelopoiesis and Suppress Inflammatory Cytokine Production

*Jeanette YC Sullivan*¹, *Helen X Huang*¹, *Jianhong C Heidmann*¹, *Francisco Diaz*¹, *Camille Brzechffa*¹, *Jane H Chen*¹, *Nneamaka Iwobi*², *Irene Hasen*³, *Amanda Ting*³, *Nicole Sparks*³, *Michael T Kleinman*³, and *Angela G Fleischman*^{1,4,5}

¹ Department of Biological Chemistry, School of Medicine, University of California, Irvine, Irvine, CA 92617, USA

² Department of Pharmaceutical Sciences, School of Pharmacy, University of California, Irvine, CA 92617, USA

³ Department of Environmental and Occupational Health, College of Health Sciences, University of California, Irvine, CA 92617, USA

⁴ Division of Hematology/Oncology, School of Medicine, University of California, Irvine, CA 92617, USA

⁵ Chao Family Comprehensive Cancer Center, University of California, Irvine, Irvine, CA 92617, USA

ABSTRACT

Tobacco and nicotine use remain the leading preventable drivers of cancer risk, and both direct and secondhand exposure to combustible cigarettes or electronic nicotine devices perturbs immune function and hematopoiesis. Here, we evaluate the impact of e-cigarette vapor and combustible cigarette smoke on *in vitro* cell inflammatory responses and *in vivo* long-term hematopoietic differentiation. In cell-based studies, cigarette smoke extract (CSE) and e-cigarette vapor extract (EVE) consistently suppress LPS-induced TNF- α secretion across macrophage/monocyte models, including primary mouse and human cells and complementary cell lines, indicating a reproducible immunosuppressive effect on mature myeloid cells. Brief *ex vivo* exposure to CSE also alters myeloid subset composition and modifies the proliferative behavior of Tet2-knockout cells, suggesting that smoke-related cues can reshape competitive dynamics among mutant and wild-type myeloid progenitors. To assess consequences of smoking behavior *in vivo*, we used a custom nose-cone inhalation system to deliver controlled exposures to combustible cigarette smoke or e-cigarette aerosol to mice. Chronic exposure increased myeloid proliferation, consistent with smoking behavior inducing premature aging of the hematopoietic stem cell pool. Taken together, these results support a model in which tobacco exposures blunt innate immune responsiveness while simultaneously driving myeloid expansion conditions that accelerate hematopoietic aging.

Keywords: e-cigarettes; macrophages; hematopoietic stem cells; TET2

INTRODUCTION

Combustible cigarette smoke and electronic cigarette aerosol are now two of the most common inhaled exposures altering immunity and blood formation. Both deliver oxidants and bioactive chemicals that can rewire inflammatory signaling and reshape marrow output, but their effects are context dependent as their impact can vary with dose, timing, and cell type under study. The hematopoietic system contains a wide variety of cells, each with unique physiological functions. At one end of the hierarchy, mature macrophages orchestrate front-line defense and repair; at the other, hematopoietic stem cells (HSCs) integrate inflammatory cues and decide whether to self-renew or differentiate. Tobacco smoke modulates the immune system across the hematopoietic hierarchy, from stem and progenitor cells to mature myeloid populations (Ramanathan et al. 2020), yet side-by-side comparisons of combustible versus e-cigarette exposures across macrophages and HSCs remain limited.

Evidence indicates tobacco smoke exerts bidirectional immune effects; it can provoke inflammation yet also blunt host defense. Smokers show elevated plasma cytokines (Elisia et al. 2020), and a smoking history worsens inflammatory and autoimmune disease courses (Arnson et al. 2010; Mahmoudzadeh et al. 2023). In the lung, smokers have 2-3-fold as many macrophages in their bronchoalveolar lavage fluid (Gonçalves et al. 2011). Paradoxically, smoking also increases the risk of infection (Lugade et al. 2014), likely reflecting impaired adaptive immune responses compromised by chronic inflammation.

Combustible cigarette smoking induces inflammation and oxidative stress and contributes to the pathogenesis of cancers and other diseases; however, the long-term carcinogenic effect of e-cigarettes has yet to be completely recognized. Combustible cigarettes contain tobacco and fillers

that are burned and release over 7000 chemicals (Zarcone et al. 2023). E-cigarettes, in contrast, are battery-operated devices that vaporize “e-liquid” containing propylene glycol (PG), vegetable glycerin (VG), flavorings, and nicotine. The accessibility, flavors, and discreet use of e-cigarettes have led to rising nicotine use among adolescents, young adults, and non-smokers. In vitro studies of cigarette smoke extract (CSE), e-cigarette vapor extract (EVE), or their components alone have produced conflicting results, showing both enhanced and repressed cytokine production by monocytes and macrophages. The heterogeneity of e-cigarette formulations further complicates these context-dependent effects. While considerable effort has been devoted to studying the impact of smoking and vaping on mature immune cells, much less is known about how these exposures affect hematopoietic stem and progenitor cells. To address these gaps, we established a standardized in vitro platform to compare the effects of CSE and EVE across multiple macrophage models and to extend these analyses to hematopoietic progenitors.

Cigarette smoke is also strongly associated with the development of clonal hematopoiesis of indeterminate potential (CHIP), the presence of mutant hematopoietic cells without blood count abnormalities (Levin et al. 2022; Dawoud et al. 2020; Kessler et al. 2022; Lee et al. 2024). CHIP occurs when HSCs acquire a mutation that provides a proliferative advantage over their normal counterparts (Marston et al. 2024). The incidence of CHIP increases with age and can be found in about 10% of the population by age 70 (Marston et al. 2024). CHIP increases the risk of blood cancers and cardiovascular diseases (Jakubek et al. 2023; Marnell et al. 2021; Schuermans and Honigberg 2025). Analysis of the UK Biobank (UKB) cohort (n=502,524) identified a significant association ($p<0.0005$) between smoking behavior and incidence of CHIP (Levin et al. 2022). A separate UKB analysis confirmed this link, showing that myeloid clonal hematopoiesis was particularly enriched among current smokers compared with past smokers (Dawoud et al. 2020).

Similarly, exome sequencing from the Geisinger MyCode Community Health Initiative revealed that heavy smoking was strongly associated with CHIP carrier status (Kessler et al. 2022). Importantly, across both the UKB and Geisinger cohorts, CHIP carriers showed an increased risk of hematologic cancers, particularly myeloproliferative neoplasms (Kessler et al. 2022). Together, these findings underscore a consistent epidemiologic link between smoking behavior and aberrant clonal hematopoiesis, but the mechanisms driving this association remain poorly understood.

The most common CHIP mutations occur in the DNMT3A, TET2, ASXL1, and JAK2 genes, which also drive the development of myeloid cancers (Xie and Zeidan 2023). These CHIP clones emerge because they gain a competitive advantage under hematopoietic stress and aging. At the stem and progenitor level, inflammatory and oxidative stress cues transiently push HSCs into cycle and bias lineage output toward the myeloid fate; chronic or repeated stress can erode self-renewal and remodel progenitor composition (Pietras 2017). TET2, an epigenetic regulator of DNA methylation, is the second most frequently mutated CHIP gene (Moran-Crusio et al. 2011; Abdel-Wahab et al. 2012; Cobo et al. 2022). Somatic *TET2* mutations can be found in 50% of chronic myelomonocytic leukemia, 30% of patients with myelodysplastic syndromes or myeloproliferative neoplasms, and are associated with worse outcomes in acute myeloid leukemia (AML) (Moran-Crusio et al. 2011). The transformation of underlying CHIP mutations into official malignancies is a key clinical concern, but the factors that control their emergence remain poorly understood. Here, we leverage the associations between CHIP, inflammation, and smoking to investigate how these factors contribute to mutant TET2 hematopoiesis.

MATERIALS AND METHODS

Mice

All animal procedures were performed under the approval of the Institutional Animal Care and Use Committee at the University of California, Irvine. Wildtype C57BL/6J (Stock No. 000664) and Tet2^{-/-} mice (Stock No. 023359) were purchased from The Jackson Laboratory (Bar Harbor, ME) at 8- to 10-weeks old and housed in specific pathogen-free facilities on a 12-hour light/dark cycle. Euthanasia was performed using an overdose of isoflurane, and euthanasia was confirmed by cervical dislocation. Where indicated, complete blood counts were obtained using the automated cell counter machine (ABCVet Hemalyzer, scilVet).

Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected from healthy blood donors in EDTA tubes. Peripheral blood mononuclear cells (PBMCs) were isolated using the LymphoprepTM density gradient medium (Stemcell).

THP-1 and RAW264.7 cells

RAW 264.7 cell lines were obtained commercially. Cells were revived from a previously frozen third passage stock and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (R10) in 10 mm tissue culture-treated Petri dishes. Cultures were maintained at 37°C in a 5% CO₂ incubator. Once the cells reached confluency, they were passaged using 1 mL of trypsin for 5 minutes to facilitate detachment. Cells were gently scraped and transferred to a 50 mL conical tube containing 40 mL of R10. From this, 10 mL of the cell suspension was replated into a fresh 10 mm Petri dish for continued culture. The remaining

cells were seeded into 96-well tissue culture-treated plates and allowed to adhere overnight in the incubator before stimulation. Cells were continuously passaged from passage 3 through passage 15.

THP-1 cell lines were obtained commercially (American Type Culture Collection, USA). Cells were thawed from a previously frozen stock and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (R10) in T-25 flasks. Cells were maintained at 37°C in a 5% CO₂ incubator. Cells were continually passaged from passage 3 through 15.

Bone marrow-derived macrophages (BMDMs)

Bone marrow was extracted by flushing the femurs and tibias with 10 mL of staining buffer using a syringe through a 45 µm mesh strainer. The collected marrow was subjected to red blood cell lysis using ACK buffer on ice for 20 minutes, followed by washing and centrifugation. The resulting cell pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (D10). A small aliquot of the suspension was analyzed by flow cytometry to determine the total bone marrow cell count. To generate bone marrow-derived macrophages (BMDMs), 20 million bone marrow cells were resuspended in 10 mL of D10 supplemented with 30% L929 cell-conditioned media. Cells were seeded in non-tissue culture-treated 10 mm Petri dishes and incubated at 37°C in a 5% CO₂ atmosphere. After 3 days, each dish was supplemented with an additional 3 mL of fresh D10 + 30% L929 media. On day 5, media in each dish was replaced entirely with 10mL fresh D10 + 30% L929 media. After 7 days, the supernatant was discarded, and the macrophages were incubated with 1 mL of trypsin for 5 minutes. Macrophages were detached by scraping gently and collecting into D10.

Cigarette smoke extract (CSE) preparation

Cigarette smoke extract (CSE) was prepared from mainstream cigarette smoke using a standard protocol. Briefly, mainstream smoke from one 2R4F research cigarette was drawn into serum-free media by continuous negative pressure and filter-sterilized to obtain 100% CSE. Volume/volume percentage (v/v %) dilutions were used for in vitro exposures.

E-cigarette vape extract (EVE) preparation

E-cigarette smoke extract (EVE) was prepared using the Naked brand Melon flavored e-liquid (65:35 VG:PG with 6mg nicotine) in a Vaporesso XROS 3 Pod System. The vape liquid was purchased from a local shop in Irvine, CA. The Vaporesso device was attached to an impinger, which is attached to the vacuum port of a tissue culture hood. To mimic inhalation, the vacuum was turned on for 4.3 seconds every minute for a duration of 75 minutes, drawing the e-cigarette vapor into 25mL of media. This protocol was developed based on the collection methods described in Williams et. al. (Williams et al. 2019).

Nose-only inhalation exposures

Exposures were performed at the Air Pollution Health Effects Laboratory at the University of California, Irvine. Exposures occurred 2 hours per day, 4 days/week, for 8 weeks. Between exposures, the mice were housed 4 per cage in an atmosphere-controlled room on a 12-hr light/dark cycle in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal housing facility at the University of California, Irvine vivarium. Aerosols were generated using a 2-s puff with a 60-s interval between puffs (35 mL puff volume) based on the ISO standard cigarette puff protocol (ISO 3308:2012) using a custom-built smoking

system (Figure 9B). This system uses a peristaltic pump to draw in aerosolized smoke from a lit combustion cigarette (1R6F Certified Reference Cigarette, Center of Tobacco Reference Products (CTRP), University of Kentucky) and directs it into a nose-only exposure manifold (In-Tox Products, Moriarty, NM, USA). For the e-cigarette exposure, the peristaltic pump draws in aerosolized vapor from a Nichrome 70-watt E-cigarette. The e-liquid used was made by mixing equal volumes of propylene glycol (Millipore, Burlington, MA) and vegetable glycerin (Fisher Chemical, Hampton, NH) with 1.5% of nicotine (ThermoFisher, Waltham, MA). Control mice were exposed to air purified over potassium permanganate-impregnated alumina beads, activated carbon, and high-efficiency particulate air (HEPA) filters, concurrently with the combustion cigarette or e-cigarette exposures.

The following information was previously published and is provided here for convenience (Ramanathan et al. 2023). The nose-cone inhalation exposure system is advantageous over whole-body exposure systems because it minimizes fur contamination and potential non-relevant exposure due to routine grooming that occurs in a whole-body exposure regime. During exposures, animals were held in individual exposure tubes that were connected to the exposure manifold and positioned with just the snout exposed to the exposure atmosphere. Exhaust ports surrounding each nose cone were under slight negative pressure to direct the flow of fresh aerosols to the animal's breathing zone and exhaust exhaled air, thereby minimizing the potential for re-breathing secondary vapor and preventing CO₂ buildup.

Bone marrow transplantation

Fresh bone marrow was harvested from euthanized adult mice. Recipient mice were lethally irradiated with a single dose of 8Gy and transplanted retro-orbitally with approximately 1.2×10^6

whole bone marrow cells. For the competitive wild-type (WT) and Tet2^{-/-} transplant, mice were transplanted with a ratio of 93% WT to 7% Tet2^{-/-} cells.

Platelet counting

Platelet counting was performed as adapted from Masters & Harrison, 2014 (Masters and Harrison 2014). Peripheral blood from the saphenous vein was mixed 1:1 with EDTA, diluted in staining buffer with 0.2 microliters of FITC CD61, and incubated for 20 minutes. Samples were further diluted and run on a Novocyte 3000 flow cytometer (Agilent Technologies) to obtain absolute platelet counts.

Flow cytometry of mouse peripheral blood/bone marrow

To determine peripheral blood cell frequencies and chimerism, red blood cells were lysed with ammonium-chloride-potassium (ACK) buffer. Cells were stained with anti-mouse antibodies specific to CD45.1, CD45.2, CD11b, CD19, CD11b, Ly6G, CD3, CD4, CD8, and Ly6C. For the immature stem and progenitor populations, bone marrow was stained with anti-mouse antibodies CD34, CD150, CD16/32, CD48, cKit (CD117), lineage cocktail (containing: CD3, Ly-6C, CD11b, CD45R, Ter-119), and Sca-1. All antibodies were used at a 1:500 dilution factor as recommended.

Colony formation assay

Colony formation assays were performed as indicated by StemCell Technology protocols. Whole bone marrow cells from WT and Tet2^{-/-} were incubated with increasing concentrations of cigarette smoke extract (CSE) or e-cigarette vape extract (EVE) overnight. Cells were washed thoroughly and plated at a concentration of 10,000 cells/ml in Methocult medium (M3224, StemCell) with IL-3 at 10ng/mL and SCF at 50ng/mL. Colonies were counted 7-12 days later.

Measurement of cytokine secretion

Concentrations of TNF- α were determined in conditioned media supernatant from cells incubated with 10ng/mL lipopolysaccharide (LPS, Sigma) and increasing concentrations of CSE and EVE for 24 hours. Samples were frozen at -80°C until analysis. Commercial enzyme-linked immunosorbent assay (ELISA) kits for human TNF- α (88-7346-88, Invitrogen/ThermoFisher) and mouse TNF- α (88-7324-88, Invitrogen/ThermoFisher) and used in accordance with the manufacturer's instructions.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM). GraphPad Prism (GraphPad Software, San Diego, CA) was used for all statistical analyses. Differences between mean values within groups were determined using ANOVA or unpaired t-tests where indicated.

RESULTS

Standardized in vitro model reveals differential inhibitory effects of cigarette smoke and e-cigarette exposure on macrophage TNF- α responses

To investigate how smoke or e-cigarette exposure influences inflammatory responses, we established a standardized workflow in which all cell culture and analytic protocols were consistent across experiments. Single-use aliquots of the original batches of CSE and EVE, ensuring that the only variable was the cell type under study. This approach enabled a direct, side-by-side comparison of different monocyte and macrophage models. Because higher concentrations of CSE and EVE can impair cell proliferation and viability, we selected minimal concentrations (0.5-2%), which did not induce significant cell death under our culture conditions.

We first examined how CSE and EVE influence tumor necrosis factor-alpha (TNF- α) production in response to lipopolysaccharide (LPS). Cells were exposed to CSE and/or EVE concurrently with LPS stimulation, and supernatant was collected 24 hours later to quantify TNF- α production by ELISA. To broadly evaluate these effects, we tested both human and murine macrophage cell lines, as well as primary macrophages derived from human peripheral blood monocytes and mouse bone marrow (Figure 1). In RAW264.7 cells, a murine macrophage cell line widely used in immunology and toxicology studies, CSE significantly suppressed TNF- α secretion in a dose-dependent manner (Figure 1A). Suppression of TNF- α by CSE was also confirmed using intracellular cytokine staining (Supplemental Figure 1). In contrast, EVE prepared from a commercially available melon-flavored e-liquid did not significantly alter TNF- α levels (Figure 1A).

We then applied the in vitro smoke and e-cigarette exposure protocol to THP-1-derived macrophages, a human monocytic cell line frequently used in immunology and oncology research. THP-1 monocytes were differentiated into macrophages with Phorbol 12-myristate 13-acetate (PMA) before stimulation. Like RAW264.7 cells, THP-1 macrophages showed a dose-dependent reduction in TNF- α secretion in response to CSE (Figure 1B). While EVE alone did not significantly suppress TNF- α production, co-exposure with CSE further reduced secretion compared with CSE alone (Figure 1B).

We next tested how CSE and EVE affect primary bone marrow-derived macrophages (BMDMs). To assess genotype-specific responses, we generated BMDMs from wild-type C57BL/6 mice and from Tet2^{-/-} mice, given that clonal hematopoiesis, most commonly involving TET2 mutations, is enriched in smokers. This design enabled us to ask whether CHIP-mutant macrophages respond differently to smoke exposure than their wild-type counterparts. Like the cell line macrophages, we observed a dose-dependent inhibition of TNF- α in both WT and Tet2^{-/-} BMDM (Figure 1C, D). When directly comparing WT vs Tet2^{-/-} macrophages, the impact of smoke was greater in Tet2^{-/-} macrophages, specifically at 2% CSE and 0.5+0.5 CSE + EVE, demonstrating a differential effect of smoking on Tet2^{-/-} macrophages. These findings indicate that when a standardized protocol is used, primary macrophages and immortalized cell lines respond similarly to CSE and EVE, both demonstrating suppressive effects of CSE.

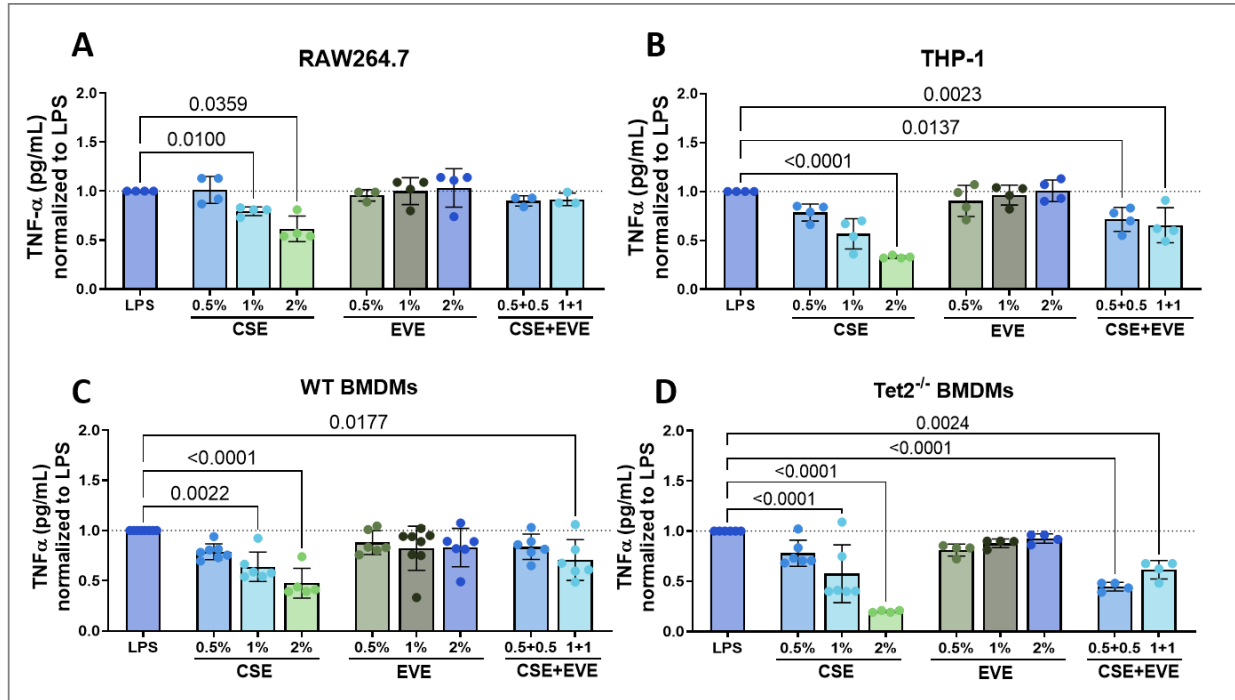


Figure 1. Cigarette smoke inhibits LPS-induced TNF- α secretion in multiple macrophage models. Adherent macrophages were washed and stimulated with 10ng/mL LPS and 0-2% CSE, 0-2% EVE, or a combination of CSE+EVE. Supernatant was collected at 24H and frozen at -80 °C until analyzed by TNF- α ELISA. Ratio of TNF- α is calculated by normalizing the amount of TNF- α secreted with CSE or EVE by the amount of TNF- α secreted by the LPS-stimulated cells alone. (A) RAW264.7 macrophages were washed, plated in 96-well plates, and incubated overnight for adherence pre-stimulation. Results are the ratios of 4 independent experiments with n=4-10/group/experiment. (B) THP-1 monocytes were plated in 96-well plates and incubated with PMA for 48 hours to induce macrophage differentiation and washed before stimulation. Results are the ratios of 4 independent experiments with n=4-10/group/experiment. (C) Bone marrow was freshly isolated from wild-type and (D) Tet2-deficient mice and incubated for 7 days with 15% L929-conditioned media (to provide mM-CSF) on non-tissue culture treated petri dishes to induce differentiation of bone-marrow-derived macrophages (BMDM). BMDMs were washed and replated prior to LPS/CSE/EVE exposure. Results are the ratios of 5-8 independent experiments with n=4-8 replicates/group/experiment. Significance is calculated by one-way ANOVA. Values represent means $\pm$ SEM.

We next applied this workflow to human peripheral blood mononuclear cells (PBMCs) freshly isolated from whole blood of healthy donors. Altogether, the responses to CSE and EVE were variable across individuals, without a uniform pattern of TNF- α inhibition or stimulation overall. However, when analyzed on a per-donor basis, the data revealed two distinct response groups: an anti-inflammatory group and a pro-inflammatory group. These groups were not associated with age, sex, or medication use, and smoking history was unavailable. Most donors (6 of 9) exhibited reduced TNF- α secretion after CSE exposure, with some also showing suppression at higher EVE doses (TNF- α ratio <1). In contrast, three donors demonstrated the opposite effect, with enhanced TNF- α secretion following exposure to CSE or EVE. These findings underscore the heterogeneity of human immune responses to smoke- and vape-derived exposures, which may reflect variability in trained immunity that shapes innate immune responsiveness.

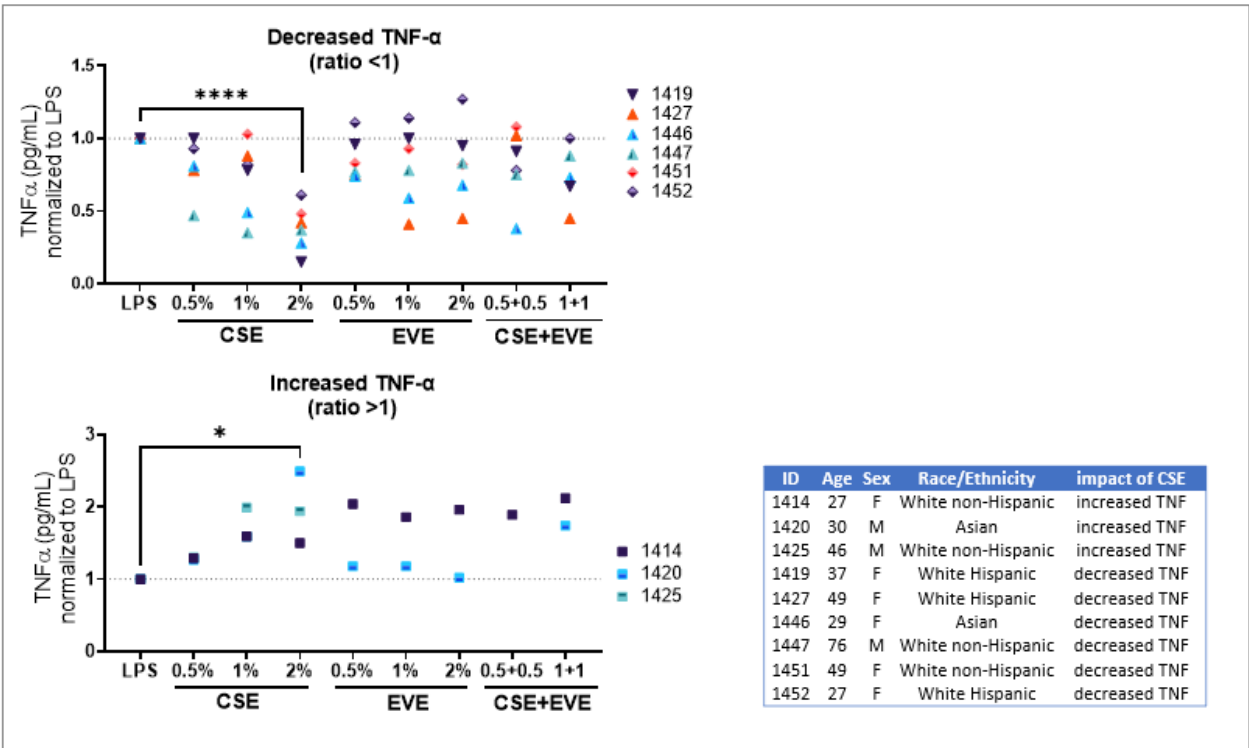
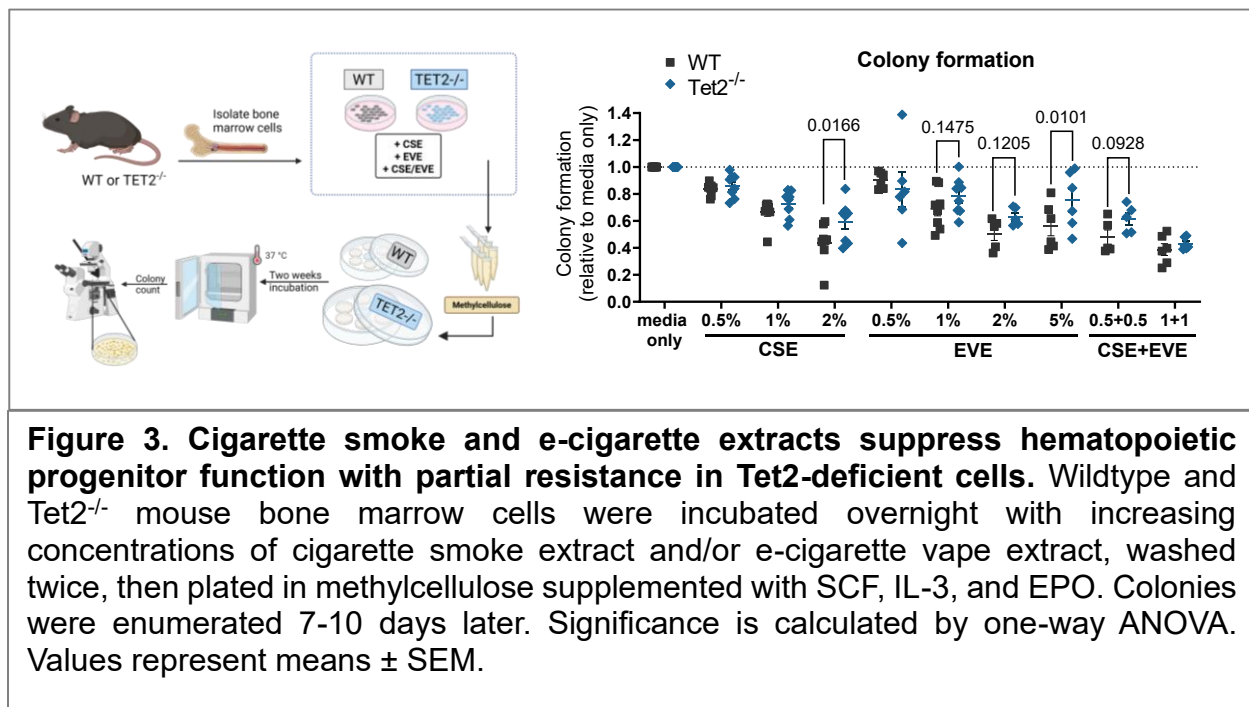


Figure 2. Human PBMCs are diverse and display either a “stimulatory” or “inhibitory” response. Normal human peripheral blood mononuclear cells were exposed to 10ng/mL LPS and 0-2% CSE, 0-2% EVE, or a combination of CSE+EVE. Supernatant was collected at 24H and frozen at -80 °C until analyzed by TNF-α ELISA. Ratio of TNF-α is calculated by normalizing the amount of TNF-α secreted with CSE or EVE by the amount of TNF-α secreted by the LPS-stimulated cells alone. Results are the mean of n=4-8 replicates per donor. Significance is calculated by one-way ANOVA. Values represent means ± SEM.

Cigarette smoke and e-cigarette extracts suppress hematopoietic progenitor function with partial resistance in Tet2-deficient cells

To examine how CSE and EVE influence hematopoietic progenitor function, we utilized methylcellulose colony formation assays to quantify myeloid progenitor activity. Given that smoking increases the risk of clonal hematopoiesis of indeterminate potential (CHIP), we tested whether progenitors carrying a common CHIP mutation, Tet2 loss-of-function, respond differently

to these exposures. Whole bone marrow from wild-type or Tet2^{-/-} mice was incubated overnight with CSE, EVE, or their combination, washed, and plated in methylcellulose. Myeloid colonies were enumerated after 7–10 days. Both CSE and EVE significantly reduced colony formation in wild-type and Tet2^{-/-} cells (Figure 3). However, Tet2^{-/-} progenitors retained higher clonogenic potential compared with wild-type cells, particularly at 1% and 2% CSE concentrations (Figure 3). These results suggest that Tet2 deficiency confers relative resistance to the suppressive effects of cigarette smoke on hematopoietic progenitors.



Evaluating the impact of CSE and EVE on the clonal competition of Tet2-deficient cells

Based on the observed effects of overnight CSE and EVE exposure on hematopoietic stem and progenitor cell function in vitro, we next investigated how this exposure impacts in vivo hematopoiesis. Given the relative resistance of Tet2^{-/-} cells in vitro, we also asked whether smoke or e-cigarette exposure might promote their expansion or alter their phenotypic behavior in vivo.

To test this, we performed competitive bone marrow transplants using cells pre-exposed to CSE, EVE, or the combination (Figure 4). Whole bone marrow cells from wild-type (CD45.1) and Tet2^{-/-} (CD45.1/2) mice were mixed at a ratio of 93:7 and cultured overnight in either R10 media only (RPMI + 10% FBS) or R10 with 0.5% CSE, 1% EVE, or 0.5% CSE and 1% EVE (Figure 4). An aliquot of cells collected after culture confirmed that the input ratio of 7% Tet2^{-/-} to 93% WT was preserved across all conditions. These co-cultured cells were then transplanted into lethally irradiated CD45.2 WT recipients (Figure 4).

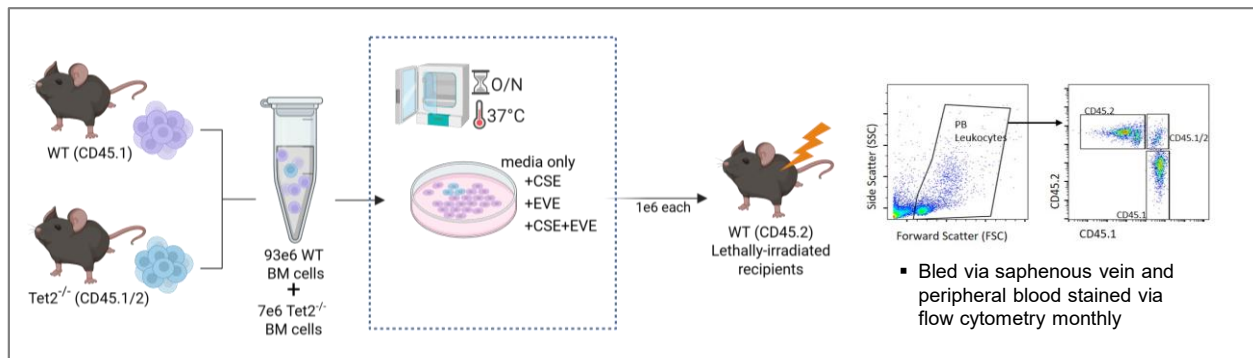


Figure 4. Experimental setup of WT: Tet2^{-/-} competitive transplant with bone marrow co-cultured overnight in smoke or e-cigarette extract. Whole bone marrow was freshly isolated from WT (CD45.1) and Tet2^{-/-} (CD45.1/2) mice and mixed at a ratio of 7% Tet2-knockout to 93% WT. The mixed cells were exposed to CSE or EVE overnight. After incubation, the cells from each were confirmed to still have 7% Tet2^{-/-} and 93% WT by flow cytometry staining (representative CD45.1, CD45.2, and CD45.1/2 gating shown). The cells were washed and transplanted into lethally irradiated CD45.2 WT mice, n=9-10 per group. Mice were bled via the saphenous vein monthly to determine peripheral blood chimerism and composition.

A single exposure to CSE or EVE reprograms HSCs toward myeloid skewing

We followed peripheral blood counts monthly following transplantation using flow cytometry (Figure 4). In the mice transplanted with cells cultured in media only, myeloid cell frequencies

remained stable over time (Figure 5A). In contrast, mice transplanted with CSE and EVE-exposed BM showed a significant ($p < 0.05$) increase in the percentage of myeloid cells over time (Figure 5A). This myeloid bias, a hallmark of HSC aging (Rossi et al. 2007), suggests that transient exposure to cigarette smoke or e-cigarette vapor can alter hematopoietic function in vivo. We further investigated specific myeloid subsets, focusing on neutrophils and monocyte/macrophages. E-cigarette vapor has been shown to impair neutrophil functions, including chemotaxis, phagocytosis, and NETosis (Corriden et al. 2020; Jasper et al. 2024). Non-exposed bone marrow cultured in media-only did not significantly increase the post-transplant proportions of neutrophils or monocytes and macrophages over time (Figure 5B). However, exposure to e-cigarette extract significantly increased the number of neutrophils in the peripheral blood (Figure 5B). In contrast, exposure to CSE or the combination of CSE+EVE increased the proliferation of monocytes and macrophages (Figure 5C). CSE also modestly increased the absolute platelet count (Figure 5D), consistent with a chronic inflammatory state. The number of lymphoid B and T cells in the peripheral blood was overall unaffected by pre-incubation with CSE or EVE. Together, these findings show that even a single pre-transplant exposure to cigarette smoke or e-cigarette extract can durably reprogram bone marrow, biasing hematopoiesis toward myelopoiesis, a hallmark feature of hematopoietic aging.

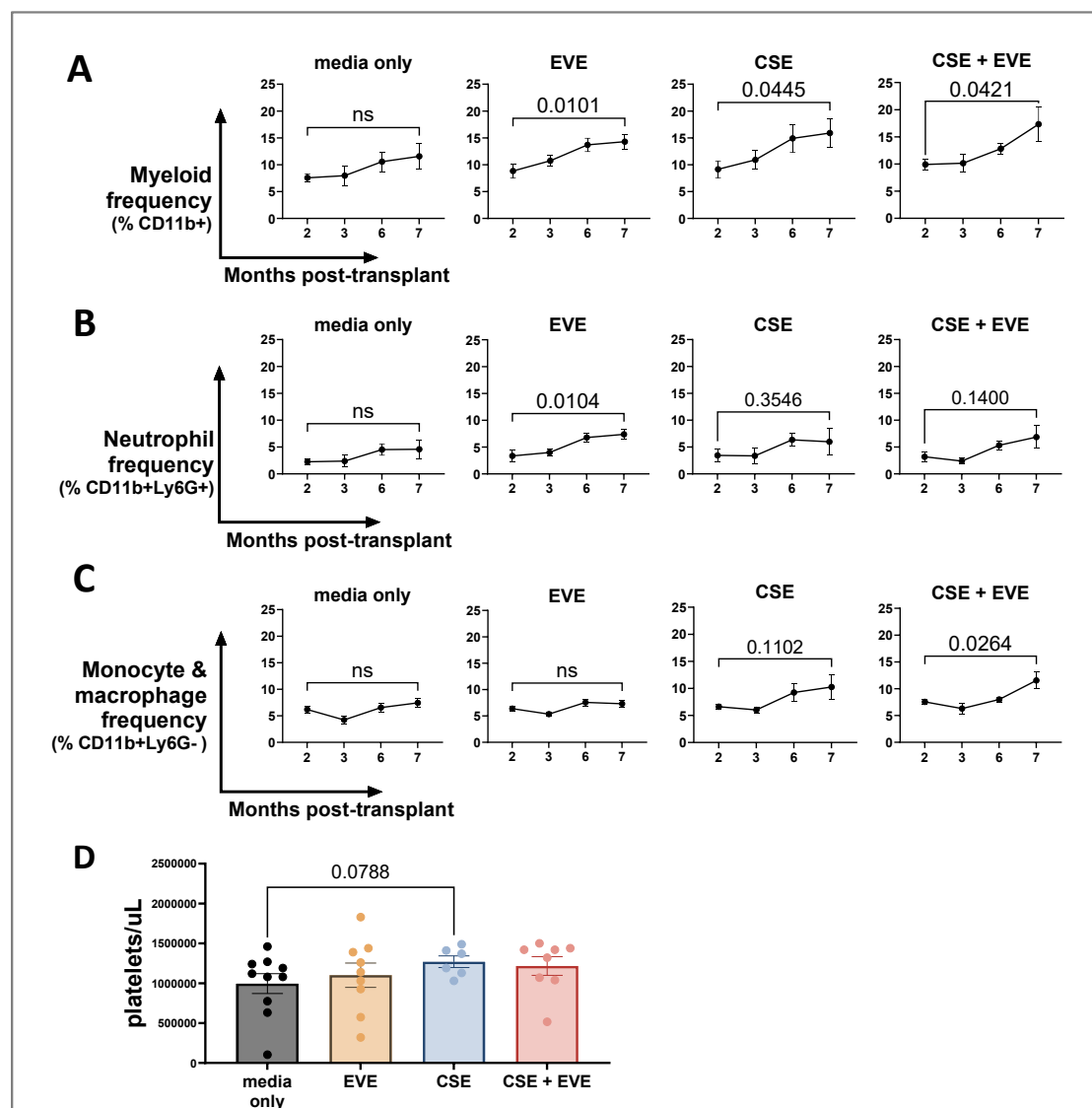


Figure 5. Exposure to CSE or EVE in HSCs induces long-term increases in circulating myeloid cell populations. Flow cytometry was used to analyze the peripheral blood myeloid and lymphoid composition of chimeric mice transplanted with CSE- or EVE-exposed cells. Changes in the frequency of (A) CD11b+CD19- myeloid cells, (B) CD11b+Ly6G+ neutrophils, and (C) CD11b+Ly6G- monocytes of all CD45+ leukocytes in the peripheral blood over time. (D) Platelet counts in the peripheral blood at month 7. Values represent means $\pm$ SEM. Significance calculated by unpaired t-tests (A-C) and one-way ANOVA (D).

EVE promotes the expansion of Tet2-deficient mutant cells in the peripheral blood

We next asked whether overnight exposure to CSE or EVE would alter the selective advantage of Tet2-deficient cells in vivo. Based on our in vitro methylcellulose data, we anticipated that smoke extract might preferentially suppress wild-type progenitors, thereby enhancing Tet2^{-/-} expansion after transplant. To test this, we measured peripheral blood chimerism monthly after competitive hematopoietic stem cell transplantation. As expected, Tet2^{-/-} cells expanded in all mice, rising from the 7% input proportion to about 30% of donor-derived cells by one month post-transplant and about 60% by two months (Figure 6A). At this point, the expansion of Tet2^{-/-} cells begins to diverge between the groups; Tet2^{-/-} continued to proliferate in recipients of cells cultured in EVE, ultimately reaching about 80% of donor-derived cells (Figure 6A). In mice transplanted with non-exposed or CSE-exposed bone marrow, Tet2^{-/-} expansion plateaued or fell slightly to around 50% (Figure 6A). These results suggest that e-cigarette aerosols provide a proliferative advantage for the clonal expansion of Tet2-deficient cells.

To analyze the impacts of CSE or EVE exposure on each of these WT or Tet2^{-/-} populations individually, we used flow cytometry to gate on the Tet2^{-/-} CD45.1/2+ and WT CD45.1+ cells of all peripheral blood CD45+ leukocytes. We then quantified the frequency of CD11b+ myeloid cells, CD11b+Ly6G+ neutrophils, and CD11b+Ly6G- monocytes (Figure 6B). Co-exposure to CSE + EVE significantly increased the proportion of myeloid cells within the Tet2^{-/-} compartment (CD45.1/2+) after 2 months (Figure 6B). At termination (10 months), the EVE- and CSE-exposed Tet2^{-/-} cells had significantly increased myeloid cell proportions (Figure 6B). These dynamics indicate that both CSE and EVE separately increase myelopoiesis of mutant Tet2^{-/-} cells, and the combination of the two accelerates this impact. In contrast, wild-type cells showed no significant

changes in overall myeloid CD11b⁺ frequencies across conditions through month 2 to termination (Figure 6B).

Closer examination of myeloid cells revealed that although the overall frequency of myeloid cells was unchanged, CSE altered the monocyte-to-neutrophil ratio. In non-exposed WT samples, myeloid cells comprised roughly 70% monocytes and 30% neutrophils, whereas CSE exposure shifted this to an even 50/50 distribution (Figure 6B). In contrast, Tet2-deficient cells displayed an opposite baseline ratio of about 60% neutrophils and 40% monocytes, which CSE further skewed to approximately 75% neutrophils and 25% monocytes (Figure 6B). Overall, EVE and CSE enhanced myelopoiesis in Tet2-deficient cells only, with CSE driving a stronger neutrophil bias. In WT cells, CSE did not change total myeloid frequency but significantly altered lineage composition, indicating that CSE affects both genotypes, whereas EVE selectively impacts Tet2-deficient cells.

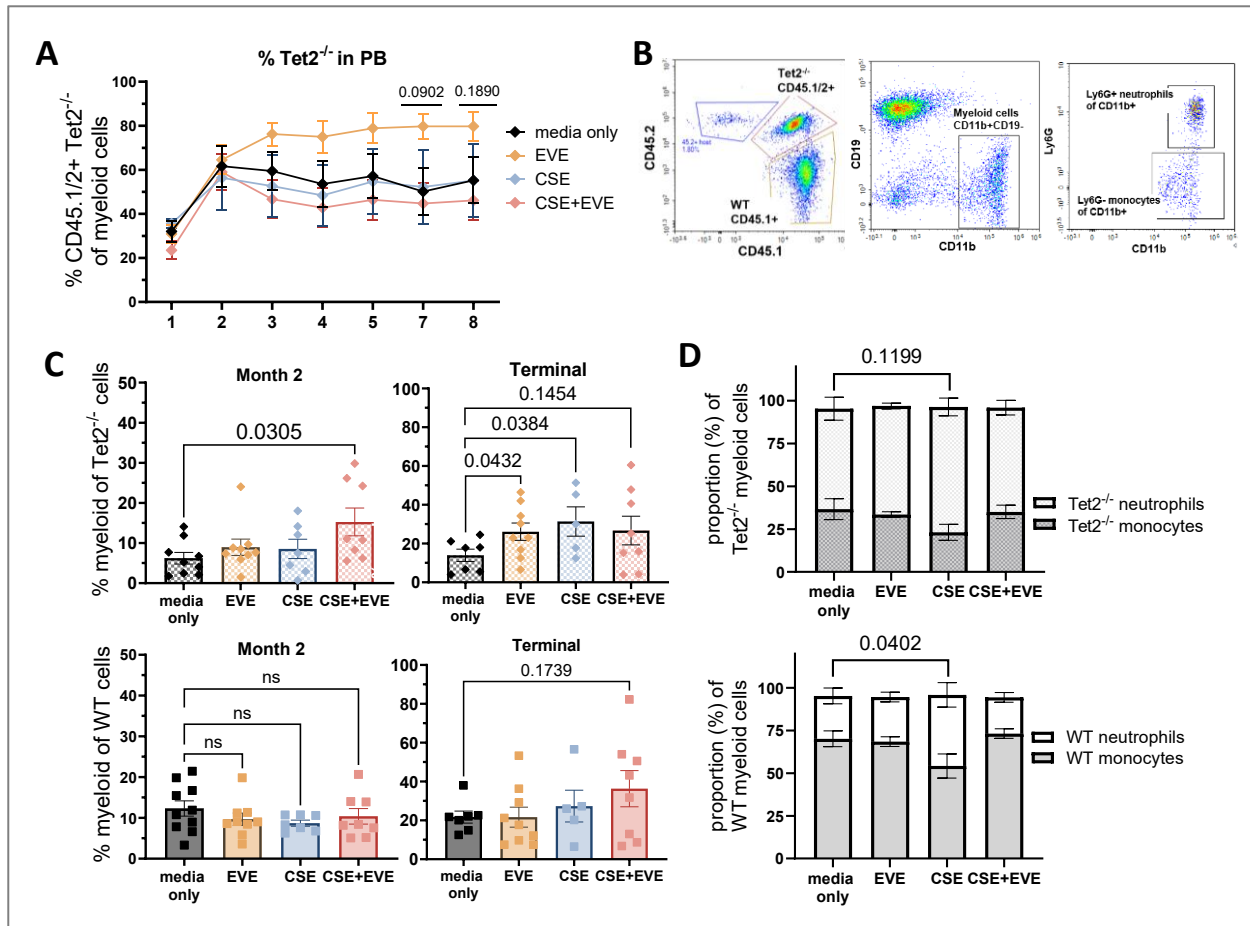


Figure 6. EVE exposure promotes the expansion of Tet2^{-/-} cells within the peripheral blood in a competitive transplant. (A) Frequency of donor-derived CD45.1/2⁺ cells of myeloid CD11b+CD19-CD45⁺ leukocytes in the peripheral blood as measured by flow cytometry gating described in (B). P-values shown are the one-way ANOVA results between the EVE and the non-exposed media-only group. (C) The percentage of CD11b+CD19- myeloid cells in Tet2^{-/-} (top) and WT (bottom) mice at months 2 (left) and month 10 at termination (right). (D) The proportions of Ly6G⁺ neutrophils and Ly6G⁻ monocytes within the CD11b+CD19- myeloid populations of the CD45.1/2⁺ Tet2^{-/-} and CD45.1 WT leukocytes. P-values shown are the results of one-way ANOVA analyses of the Ly6G⁺ neutrophil frequencies. Values represent means $\pm$ SEM.

CSE and EVE increase the proportion of Tet2-deficient inflammatory Ly6C^{++hi} monocytes

To further assess how smoke exposure influences myeloid cell function, we analyzed the distribution of monocyte subsets in the transplanted mice. Monocytes are functionally heterogeneous and are typically classified based on their surface marker expression. In mice, subsets are defined by Ly6C expression: Ly6C^{++high} monocytes correspond to classical “inflammatory” monocytes (McGrath et al. 2015; Trzebanski and Jung 2020; Li et al. 2022), which rapidly respond to immune challenges and exhibit strong phagocytic and inflammatory capacity. Ly6C-low monocytes represent the nonclassical “patrolling” subset, which contributes to vascular homeostasis, wound healing, and tissue repair (Lessard et al. 2017; Robinson et al. 2021; Wong et al. 2011). The Ly6C^{+intermediate} population has characteristics that overlap with the inflammatory phenotype of the classical subset (Wong et al. 2012). Peripheral blood monocytes of mice transplanted with non-exposed BM were composed of about 20% classical Ly6C^{++high} monocytes, 40% nonclassical Ly6C-low monocytes, and 40% Ly6C^{+intermediate} monocytes (Figure 7A). Mice transplanted with EVE- or CSE-exposed BM displayed a modest increase in inflammatory Ly6C^{++high} monocytes, at the expense of the Ly6C-low subset (Figure 7A). To assess whether these effects differed between genotypes, we examined each subset separately, comparing wild-type (CD45.1⁺) and Tet2^{-/-} (CD45.1/2⁺) donor cells (Figure 7B). The Tet2^{-/-} monocytes have a higher relative proportion of Ly6C^{++hi} monocytes than the WT monocytes (Figure 7B). Notably, the mice transplanted with HSCs exposed to EVE, CSE, or CSE+EVE had significantly higher numbers of inflammatory Ly6C^{++hi} monocytes, but selectively in the Tet2^{-/-} population (Figure 7B).

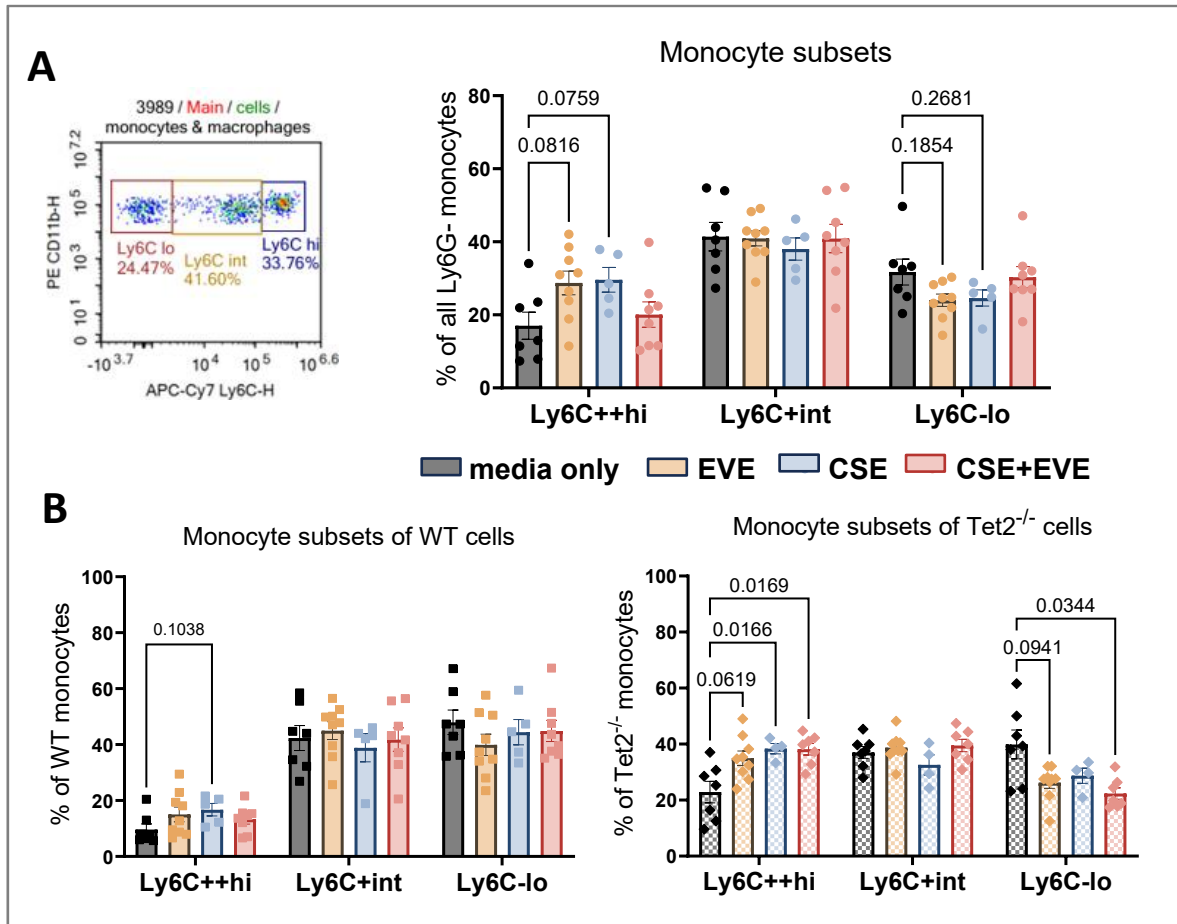


Figure 7. Impact of CSE and EVE on WT and Tet2^{-/-} monocyte subsets. (A) Monocyte subsets of chimeric mice transplanted with CSE- and EVE-exposed bone marrow as defined by relative Ly6C expression measured by flow cytometry. (B) Ly6C subsets of CD45.1 WT (left) or CD45.1/2⁺ Tet2^{-/-} (right) CD11b⁺Ly6G⁺ monocytes. Significance was determined by one-way ANOVA. Values represent means $\pm$ SEM.

EVE promotes Tet2^{-/-} proliferation in LT-HSCs in the bone marrow niche

After ten months post-transplant, bone marrow and peripheral blood were collected from the transplanted mice and stained for mature and immature stem/progenitor cell populations. Hematopoietic stem cell populations were identified by staining of the signaling lymphocyte

activation molecule (SLAM) family markers CD48 and CD150, with CD48-CD150⁺ identifying the most primitive long-term HSCs (LT-HSCs, also referred to as LSK-SLAM) (Figure 8A). We observed a significant increase in Tet2^{-/-} contribution to LKS-SLAM cells in the EVE group compared to the media alone group, demonstrating that Tet2^{-/-} LT-HSC gained a stronger selective advantage over their WT counterparts in the context of a brief ex vivo exposure to EVE (Figure 8B). This expansion of Tet2^{-/-} cells in the EVE-exposed transplant was also reflected in the peripheral blood throughout the myeloid compartment, with EVE driving proliferation of Tet2-deficient cells through the monocyte (Ly6G⁻) and neutrophil (Ly6G⁺) cell types (Figure 8C). In addition to staining for chimerism, we analyzed the frequencies of mature myeloid cells and myeloid progenitors in the bone marrow. EVE slightly increased the frequency of myeloid (CD11b⁺) cells within the bone marrow and significantly increased the neutrophil bias of these myeloid cells (Supplemental Figure 2A, B).

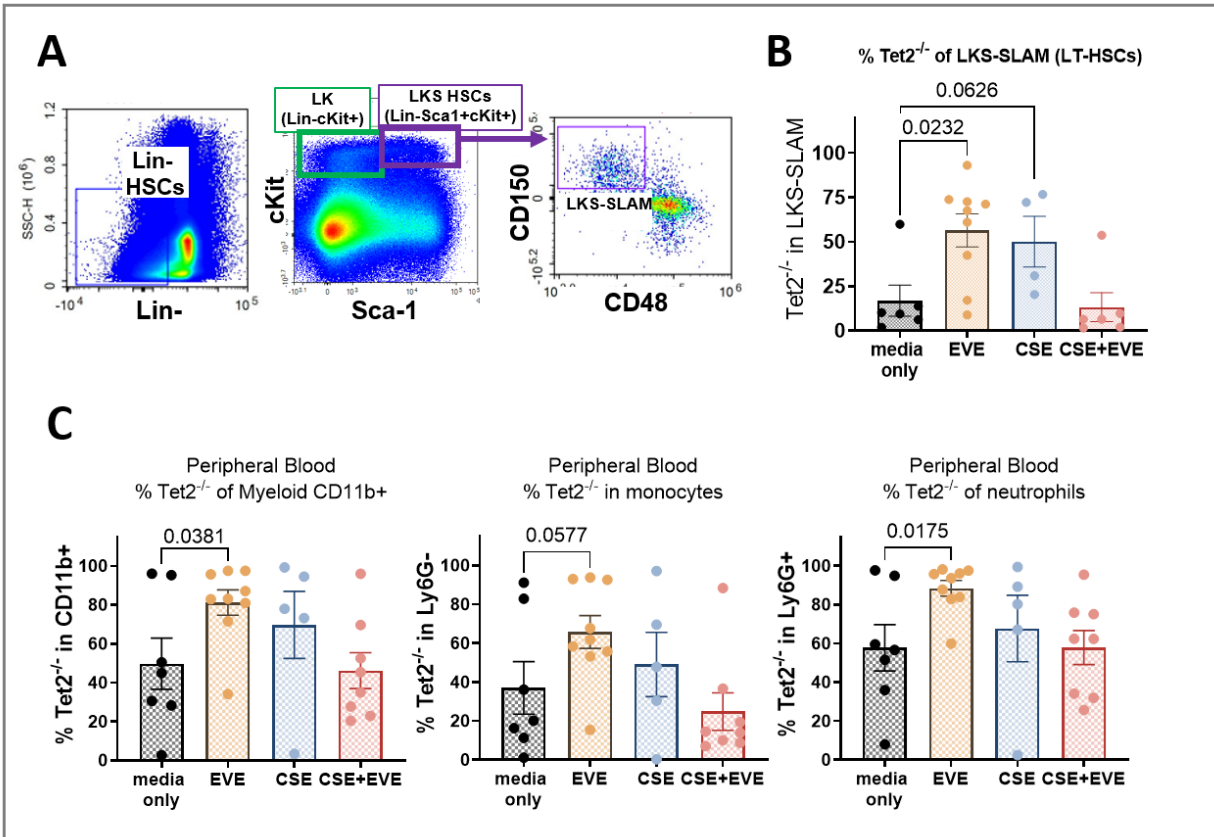


Figure 8. Tet2^{-/-} chimerism in bone marrow at ten months post-competitive transplant with CSE and EVE-exposed HSCs. (A) Representative gating of HSCs. (B) Contribution of Tet2^{-/-} cells to the LKS-SLAM (long-term HSCs) HSCs compartment at termination. (C) Contribution of Tet2^{-/-} cells of the peripheral blood myeloid compartments at termination. Significance was determined by one-way ANOVA. Values represent means $\pm$ SEM.

Cigarette smoke and e-cigarette vape consumption increase the frequency of myeloid cells in the peripheral blood

To model the effects of transitioning from combustible smoking to vaping on hematopoiesis, we exposed mice to sequential combustible smoke and e-cigarette aerosol (Figure 9). A nose-only exposure system was used to ensure uniform delivery and to avoid variability from ingestion or dermal absorption inherent to whole-body exposure (Figure 9A). Mice were exposed to

conventional cigarette smoke or clean air for 2 hours per day, 4 days per week (Figure 9B). After two months of smoke exposure, mice in the smoke group were switched to either e-cigarette vapor (n = 6) or clean air (n = 6) (Figure 9B).

Complete blood counts remained largely stable during the exposure period, except for a significant rise in platelet counts in the smoke followed by e-cigarette group (Figure 9D). The most pronounced increase occurred after the switch to e-cigarette exposure (Figure 9C–D), suggesting persistent inflammatory activation following prior smoke exposure. In addition to blood counts, we monitored myeloid and lymphoid cell frequencies in peripheral blood throughout the exposure period and found a myeloid skewing in the smoke followed by e-cigarette group (Figure 9E). At study termination, phagocytic activity of peritoneal macrophages did not differ between groups (Supplemental Figure 3), and the frequency of long-term hematopoietic stem cells in bone marrow was unchanged by either smoke or e-cigarette exposure (Supplemental Figure 3).

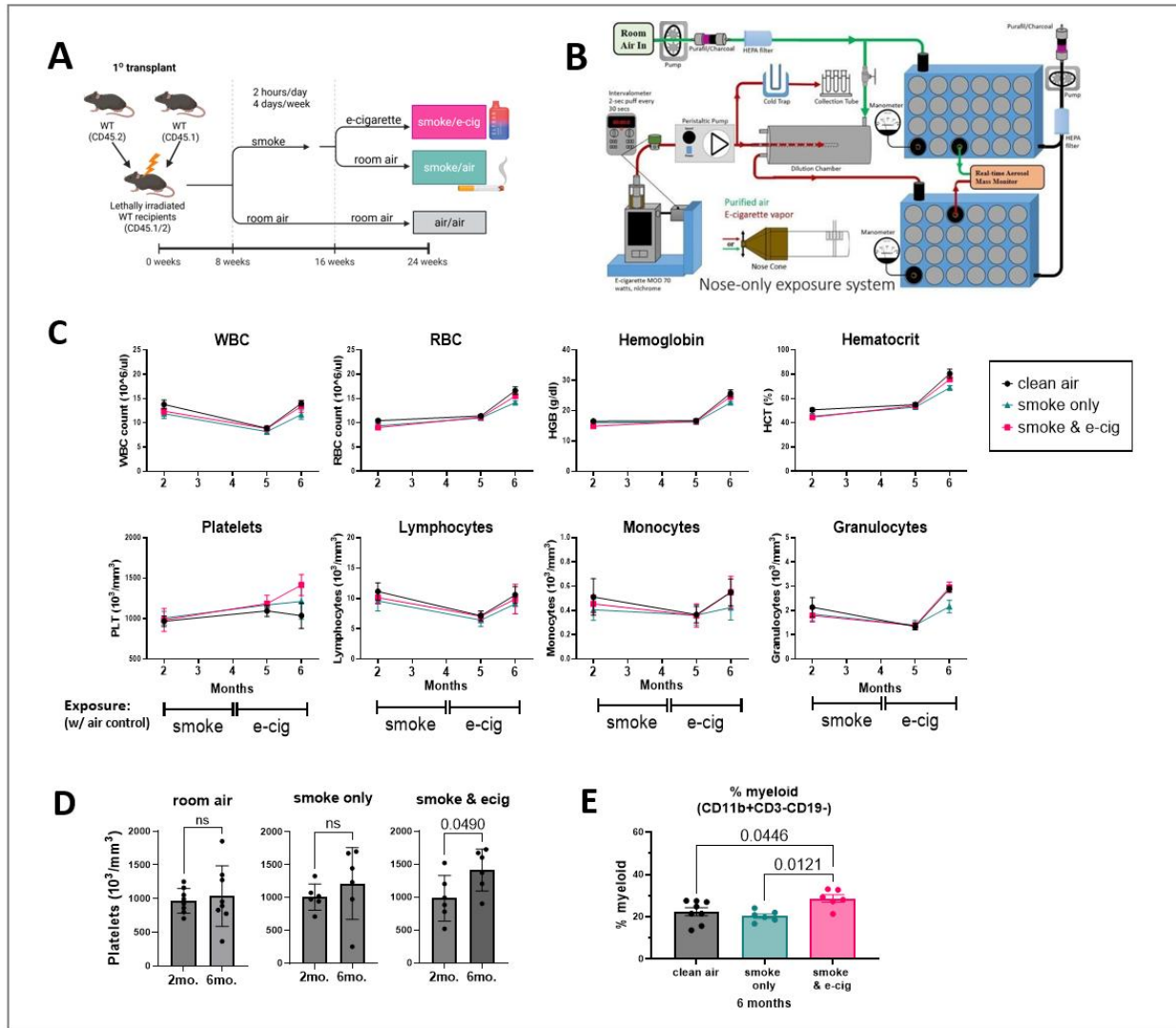


Figure 9. Long-term inhalation of traditional and e-cigarette vape smoke increases the frequency of platelets and myeloid cells in the peripheral blood. (A) Experimental setup of BM transplant and 6-month *in vivo* smoke and e-cigarette exposure. (B) Schematic of custom nose-only exposure system. (C) Complete blood counts of mice during exposure period. (D) Platelet counts at the beginning and end of exposure period. (E) Frequency of CD11b+ myeloid cells of all CD45+ leukocytes in the peripheral blood at end of exposure. Values represent means $\pm$ SEM. Significance calculated by paired t-tests (D) and one-way ANOVA (E).

Smoke exposure induces long-lasting myeloid skewing in secondary transplants

To test how smoke or e-cigarette exposure influences the long-term reconstitution capacity of hematopoietic stem cells, we performed secondary bone marrow transplants with bone marrow from the “clean air”, “smoke only”, or “smoke & e-cig” exposed mice (n = 10–13 per exposure group) (Figure 10). Both smoke-only and combined smoke & e-cigarette exposure led to increased frequencies of monocytes and macrophages (CD45⁺CD11b⁺Ly6G⁻) in the peripheral blood with age (Figure 10), consistent with a myeloid bias. Myeloid skewing is a hallmark of hematopoietic aging, and these results suggest that smoke and e-cigarette exposure imprint long-lasting changes on stem cells, which persist through serial transplantation and functionally resemble accelerated HSC aging.

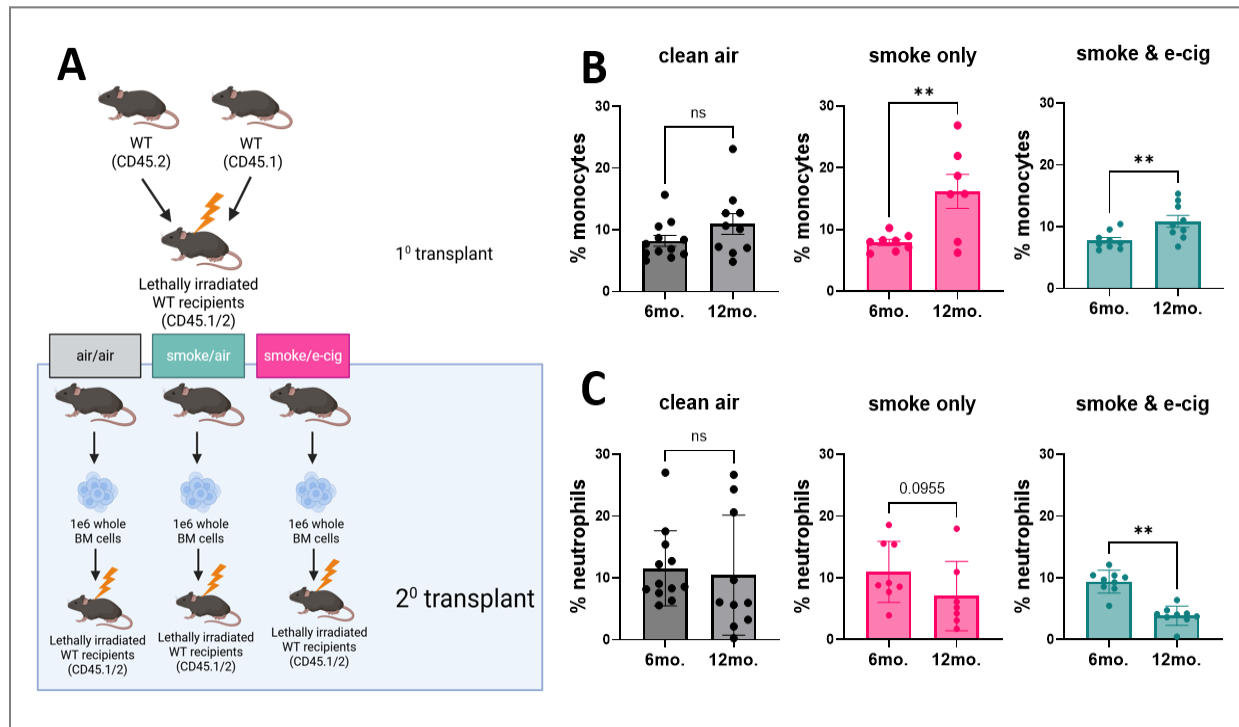


Figure 10. Exposure to cigarette smoke and e-cigarette vapor increases monocytois and decreases neutrophil differentiation in a secondary transplant. (A) Setup of BM transplant from smoke and e-cigarette-exposed mice into non-exposed mice. (B) Frequency of CD11b+Ly6G- monocytes of all CD45+ leukocytes in the peripheral blood. (C) Frequency of CD11b+Ly6G+ neutrophils of all CD45+ leukocytes in the peripheral blood. Values represent means $\pm$ SEM. Significance calculated by paired t-tests.

DISCUSSION

In this study, we examined the impact of both *in vivo* cigarette smoke and e-cigarette vape inhalation, as well as *in vitro* exposure to concentrated condensed smoke or vape extracts. Evidence suggests that e-cigarette use impacts hematopoiesis and immune cell function (Marques et al. 2021; Scott et al. 2018; Kelesidis et al. 2020). However, the literature is limited and contradictory, and there is very little work on e-cigarettes and clonal hematopoiesis specifically. Extending this investigation from primitive HSCs through the mature myeloid compartment provides insight into how e-cigarettes impact chronic inflammatory responses that can also contribute to mutant hematopoiesis. Here, we found that cigarettes and e-cigarettes increase myelopoiesis in mice and blunt TNF- α secretion in *in vitro* models of acute immune stimulation.

We first established the impacts of our prepared cigarette smoke extract (CSE) and e-cigarette vape extract (EVE) on mature myeloid cells and HSCs *in vitro*. Lipopolysaccharide (LPS), or endotoxin, is a component of the outer cell membrane of Gram-negative bacteria and is a potent activator of inflammatory responses. Based on the literature documenting increased levels of inflammatory cytokines in smokers, we anticipated increased TNF- α production in response to CSE or EVE exposure. Contrary to this hypothesis, we observed that CSE suppressed LPS-induced TNF- α secretion. We demonstrated a dose-dependent effect of CSE on human and mouse cell lines, on freshly isolated bone marrow-derived macrophages from wild-type and Tet2^{-/-} mice, and in human PBMCs. This finding encapsulates one of the central debates in nicotine research. Given the complexity of the immune system, it is perhaps unsurprising that there is evidence for both suppression (Ween et al. 2017; Hamon et al. 2024; Ouyang et al. 2000; Arimilli et al. 2017; Breivik et al. 2009; Chen et al., n.d.; Gaschler et al. 2008) and exacerbation (Zhao et al. 2023; Giebe et al. 2023; Mehta and Dhawan 2020) of immune responses by cigarettes and nicotine, depending on

the cell and context. Although the finding that CSE inhibits TNF- α seems paradoxical to the increased levels of inflammatory cytokines in smokers, our findings support the paradigm that chronic inflammation impairs an acute immune response. We posit that chronic nicotine use over time increases overall levels of circulating inflammatory cytokines but inhibits the ability to respond to an immune stimulus, hence serving both pro- and anti-inflammatory roles. This is supported by studies such as Elisia et al, who observed that whole blood from smokers has elevated numbers of monocytes and granulocytes and overall increased levels of pro-inflammatory cytokines, but an impaired immune response to an ex vivo acute immune challenge (Elisia et al. 2020). The suppression of inflammatory cytokine production may be a pathway by which cigarette smoke impairs acute immune responses.

We then sought to understand how these smoke-impaired immune responses impact the development of clonal hematopoiesis. Colony formation unit assays are the gold standard for assessing HSC function (Sarma et al. 2010). A fixed number of input hematopoietic progenitors is cultured in a semisolid medium, and the quantity and morphology of colonies formed represent HSC differentiation and proliferation ability. We demonstrated that both CSE and EVE significantly impair HSC colony formation in both WT and Tet2-deficient cells, however Tet2^{-/-} are less impacted compared to WT HSC. El-Mouelhy et al found significantly reduced CFU after exposure to cigarette smoke but not a milk and honey e-cigarette (El-Mouelhy et al. 2022). Our results are consistent with the literature on traditional combustible tobacco cigarettes and reinforce the heterogeneity of e-cigarette products and their impacts.

Studies of the health impacts of cigarette and e-cigarette smoking have naturally been primarily focused on the lungs (Seiler et al. 2020). Smoking has been shown to increase the severity of COPD (Kastratovic et al. 2024; Han et al. 2021; Gong et al. 2019). The lungs contain abundant

immune cells, especially macrophages, which originate from both embryonic and hematopoietic stem cell origins (Wohnhaas et al. 2024). Interestingly, individuals with CHIP have both an increased risk of COPD and increased disease severity (Miller et al. 2022). In fact, Miller et al. demonstrated that mice with hematopoietic Tet2 deficiency developed significantly more emphysema after 6 months of cigarette smoke exposure, notably without expansion of the Tet2^{-/-} cells. We have previously shown that 2 months of nose-only exposure to traditional combustible smoke promotes the expansion of Tet2^{-/-} cells (Ramanathan et al. 2023). In this study, we combine in vitro CSE and EVE exposures with in vivo transplants to elucidate a novel dynamic of how smoking impacts hematopoiesis. To our knowledge, this is the first study that tracks the differentiation dynamics of HSCs exposed to cigarette and e-cigarette extracts in an in vivo bone marrow transplant. Competitive BM transplants are a well-established method of assessing relative proliferation and fitness between wild-type and mutant HSCs. Using this competitive model, we demonstrate that exposure to e-cigarette vape extract provides a selective advantage to Tet2-deficient cells. Mice transplanted with EVE-exposed BM had increased frequencies of Tet2-deficient cells in the bone marrow and peripheral blood several months post-transplant. Additionally, mice transplanted with both EVE- and/or CSE-exposed BM had deranged hematopoiesis at over 9 months post-exposure, demonstrating that smoke products have enduring impacts on HSCs. The disparity in the neutrophil-to-monocyte ratio observed between Tet2^{-/-} and WT cells indicates that, in a chimeric model, Tet2^{-/-} myeloid progenitors favor neutrophil differentiation over monocyte differentiation. In contrast, the WT cells do the opposite. Cigarette smoke increased neutrophil frequencies in both WT and Tet2-deficient cells.

We identified that both CSE and EVE increase the frequency of inflammatory (Ly6C^{++hi}) classical monocytes. Characterizing monocyte heterogeneity has redefined our understanding of

these cells and identified new potential mechanisms for controlling inflammation. Human *in vivo* deuterium labeling suggests that classical monocytes leave the bone marrow first and circulate for about a day, while intermediate and nonclassical monocytes have slightly longer lifespans of ~4 and ~7 days, respectively (Patel et al. 2017). The quantity and relative frequencies are altered under pathological stressors such as infection, cancer, cardiac events, and inflammatory or autoimmune diseases (Kratofil et al. 2017). Monocyte subsets distributions differ in the peripheral blood, BM, lymph nodes, and spleen, depending on local needs (Ożańska et al. 2020). Idel et al observed that smokers have significantly increased expression of the integrin CD11b, an immune response suppressor, on their classical and intermediate monocyte subsets (Idel et al. 2022). Manipulating monocyte subsets to promote patrolling monocytes over inflammatory classical monocytes is being explored as a potential therapeutic approach for autoimmune diseases.

To extend our study, we performed long-term nose-only inhalation exposures to traditional combustible cigarette smoke followed by a switch to either room air (smoking cessation) or to e-cigarette aerosols (switching from smoking to vaping). Our findings are consistent with Verma et al., who observed increased myeloid progenitors and decreased colony-forming ability in mice exposed to cigarette smoke 3 days/week for 4 weeks (Verma et al. 2025). A key distinction between our *in vitro* CSE and EVE cell exposures and the nose-only inhalations is that the EVE is created using a commercially available melon-flavored e-liquid. In contrast, to eliminate commercial manufacturing variables, the inhaled e-cigarette aerosol is generated from house-made e-liquid containing a 50/50 PG/VG ratio and 1.5% nicotine. Including both commercial and house-made e-cigarette formulations in these studies highlights that even the base components alone alter hematopoiesis.

These long-term cellular analyses of the impact of e-cigarettes or traditional tobacco cigarettes on hematopoiesis provide insight into how smoking history may impact the progression of CHIP. We conclude that both combustible and electronic cigarette smoking derange hematopoiesis to distort myeloid differentiation. Importantly, these results suggest that the impacts of even minimal exposure to smoke extracts are persistent after smoking cessation. Although we can tell smokers to cease, we cannot erase their smoking history. Therefore, understanding how smoking impacts the body, even after cessation, is of critical importance when identifying effective therapeutics. As the population ages, incorporating studies of clonal hematopoiesis in disease contexts is of increasing clinical concern. Our results demonstrate that exposure to both cigarettes and e-cigarettes permanently changes long-term HSC myeloid lineage decisions.

AUTHOR CONTRIBUTIONS

JYCS designed and performed the experiments, analyzed the data, and wrote the manuscript; HXH, CB, FD, and JHC performed experiments; NI and NLS provided reagents; DH, IH, AT and MK developed and oversaw or performed nose-only exposures; AGF developed experimental plan, oversaw research, analyzed data, and edited the manuscript.

ACKNOWLEDGEMENTS

We thank Irene Hasen and Amanda Ting in the Air Pollution and Health Effects Laboratory at the University of California, Irvine, for their assistance with the in vivo exposures.

FUNDING

This work was supported by the Tobacco-Related Disease Research Program (TRDRP) of the University of California and UC Irvine Chao Comprehensive Cancer Center (P30CA062203).

REFERENCES

- Abdel-Wahab, Omar, Ayalew Tefferi, and Ross L. Levine. 2012. "Role of TET2 and ASXL1 Mutations in the Pathogenesis of Myeloproliferative Neoplasms." *Hematology/Oncology Clinics of North America* 26 (5): 1053–64. <https://doi.org/10.1016/j.hoc.2012.07.006>.
- Arimilli, Subhashini, Eckhardt Schmidt, Brad E. Damratoski, and G. L. Prasad. 2017. "Role of Oxidative Stress in the Suppression of Immune Responses in Peripheral Blood Mononuclear Cells Exposed to Combustible Tobacco Product Preparation." *Inflammation* 40 (5): 1622–30. <https://doi.org/10.1007/s10753-017-0602-9>.
- Arnson, Yoav, Yehuda Shoenfeld, and Howard Amital. 2010. "Effects of Tobacco Smoke on Immunity, Inflammation and Autoimmunity." *Journal of Autoimmunity, The Environment, Geoepidemiology and Autoimmune Disease*, vol. 34 (3): J258–65. <https://doi.org/10.1016/j.jaut.2009.12.003>.
- Breivik, T., Y. Gundersen, P. Gjermo, S. Von Hörsten, and P. K. Opstad. 2009. "Nicotinic Acetylcholine Receptor Activation Mediates Nicotine-Induced Enhancement of Experimental Periodontitis." *Journal of Periodontal Research* 44 (3): 297–304. <https://doi.org/10.1111/j.1600-0765.2009.01223.x>.
- Chen, Haiyan, Mark J Cowan, Jeffrey D Hasday, Stefanie N Vogel, and Andrei E Medvedev. n.d. *Tobacco Smoking Inhibits Expression of Proinflammatory Cytokines and Activation of IL-1R-Associated Kinase, P38, and NF- B in Alveolar Macrophages Stimulated with TLR2 and TLR4 Agonists*.
- Cobo, Isidoro, Tiffany Tanaka, Christopher K. Glass, and Calvin Yeang. 2022. "Clonal Hematopoiesis Driven by DNMT3A and TET2 Mutations: Role in Monocyte and Macrophage Biology and Atherosclerotic Cardiovascular Disease." *Current Opinion in Hematology* 29 (1): 1–7. <https://doi.org/10.1097/MOH.0000000000000688>.
- Corriden, Ross, Alexander Moshensky, Christine M. Bojanowski, et al. 2020. "E-Cigarette Use Increases Susceptibility to Bacterial Infection by Impairment of Human Neutrophil Chemotaxis, Phagocytosis, and NET Formation." *American Journal of Physiology-Cell Physiology* 318 (1): C205–14. <https://doi.org/10.1152/ajpcell.00045.2019>.
- Dawoud, Ahmed A. Z., William J. Tapper, and Nicholas C. P. Cross. 2020. "Clonal Myelopoiesis in the UK Biobank Cohort: ASXL1 Mutations Are Strongly Associated with Smoking." *Leukemia* 34 (10): 10. <https://doi.org/10.1038/s41375-020-0896-8>.
- Elisia, Ingrid, Vivian Lam, Brandon Cho, et al. 2020. "The Effect of Smoking on Chronic Inflammation, Immune Function and Blood Cell Composition." *Scientific Reports* 10 (1): 19480. <https://doi.org/10.1038/s41598-020-76556-7>.
- El-Mouelhy, Abir Tarek Mansour, Sherine Adel Nasry, Omnia Abou El-Dahab, Dina Sabry, and Karim Fawzy El-Sayed. 2022. "In Vitro Evaluation of the Effect of the Electronic Cigarette Aerosol, Cannabis Smoke, and Conventional Cigarette Smoke on the Properties of

- Gingival Fibroblasts/Gingival Mesenchymal Stem Cells.” *Journal of Periodontal Research* 57 (1): 104–14. <https://doi.org/10.1111/jre.12943>.
- Gaschler, Gordon J., Caleb C. J. Zavitz, Carla M. T. Bauer, et al. 2008. “Cigarette Smoke Exposure Attenuates Cytokine Production by Mouse Alveolar Macrophages.” *American Journal of Respiratory Cell and Molecular Biology* 38 (2): 218–26. <https://doi.org/10.1165/rcmb.2007-0053OC>.
- Giebe, Sindy, Melanie Brux, Anja Hofmann, et al. 2023. “Comparative Study of the Effects of Cigarette Smoke versus Next-Generation Tobacco and Nicotine Product Extracts on Inflammatory Biomarkers of Human Monocytes.” *Pflügers Archiv - European Journal of Physiology* 475 (7): 823–33. <https://doi.org/10.1007/s00424-023-02809-9>.
- Gonçalves, R. B., R. D. Coletta, K. G. Silvério, et al. 2011. “Impact of Smoking on Inflammation: Overview of Molecular Mechanisms.” *Inflammation Research* 60 (5): 409–24. <https://doi.org/10.1007/s00011-011-0308-7>.
- Gong, Jiannan, Hui Zhao, Tanzhen Liu, et al. 2019. “Cigarette Smoke Reduces Fatty Acid Catabolism, Leading to Apoptosis in Lung Endothelial Cells: Implication for Pathogenesis of COPD.” *Frontiers in Pharmacology* 10 (August). <https://doi.org/10.3389/fphar.2019.00941>.
- Hamon, Rhys, Leigh Thredgold, Asiri Wijenayaka, Nicole Anne Bastian, and Miranda P. Ween. 2024. “Dual Exposure to E-Cigarette Vapour and Cigarette Smoke Results in Poorer Airway Cell, Monocyte, and Macrophage Function Than Single Exposure.” *International Journal of Molecular Sciences* 25 (11): 11. <https://doi.org/10.3390/ijms25116071>.
- Han, Hongwei, Guangda Peng, Maureen Meister, et al. 2021. “Electronic Cigarette Exposure Enhances Lung Inflammatory and Fibrotic Responses in COPD Mice.” *Frontiers in Pharmacology* 12 (July). <https://doi.org/10.3389/fphar.2021.726586>.
- Idel, Christian, Kristin Loyal, Dirk Rades, et al. 2022. “Smoking-, Alcohol-, and Age-Related Alterations of Blood Monocyte Subsets and Circulating CD4/CD8 T Cells in Head and Neck Cancer.” *Biology* 11 (5): 658. <https://doi.org/10.3390/biology11050658>.
- Jakubek, Yasminka A., Alexander P. Reiner, and Michael C. Honigberg. 2023. “Risk Factors for Clonal Hematopoiesis of Indeterminate Potential and Mosaic Chromosomal Alterations.” *Translational Research* 255 (May): 171–80. <https://doi.org/10.1016/j.trsl.2022.11.009>.
- Jasper, Alice E., Aduragbemi A. Faniyi, Lauren C. Davis, et al. 2024. “E-Cigarette Vapor Renders Neutrophils Dysfunctional Due to Filamentous Actin Accumulation.” *Journal of Allergy and Clinical Immunology* 153 (1): 320-329.e8. <https://doi.org/10.1016/j.jaci.2023.08.025>.
- Kastratovic, Nikolina, Ivan Cekerevac, Vanesa Sekerus, et al. 2024. “Effects of Combustible Cigarettes and Heated Tobacco Products on Immune Cell-Driven Inflammation in Chronic Obstructive Respiratory Diseases.” *Toxicological Sciences* 200 (2): 265–76. <https://doi.org/10.1093/toxsci/kfae068>.

- Kelesidis, Theodoros, Elizabeth Tran, Sara Arastoo, et al. 2020. "Elevated Cellular Oxidative Stress in Circulating Immune Cells in Otherwise Healthy Young People Who Use Electronic Cigarettes in a Cross-Sectional Single-Center Study: Implications for Future Cardiovascular Risk." *Journal of the American Heart Association* 9 (18): e016983. <https://doi.org/10.1161/JAHA.120.016983>.
- Kessler, Michael D., Amy Damask, Sean O'Keeffe, et al. 2022. "Common and Rare Variant Associations with Clonal Haematopoiesis Phenotypes." *Nature* 612 (7939): 301–9. <https://doi.org/10.1038/s41586-022-05448-9>.
- Kratofil, Rachel M., Paul Kubes, and Justin F. Deniset. 2017. "Monocyte Conversion During Inflammation and Injury." *Arteriosclerosis, Thrombosis, and Vascular Biology* 37 (1): 35–42. <https://doi.org/10.1161/ATVBAHA.116.308198>.
- Lee, Jung-Kyu, Hongyul An, Youngil Koh, and Chang-Hoon Lee. 2024. "Clonal Hematopoiesis of Indeterminate Potential Is Associated with Current Smoking Status and History of Exacerbation in Patients with Chronic Obstructive Pulmonary Disease." *Tuberculosis and Respiratory Diseases*, ahead of print, February 6. <https://doi.org/10.4046/trd.2023.0165>.
- Lessard, Anne-Julie, Manon LeBel, Benoit Egarnes, et al. 2017. "Triggering of NOD2 Receptor Converts Inflammatory Ly6Chigh into Ly6Clow Monocytes with Patrolling Properties." *Cell Reports* 20 (8): 1830–43. <https://doi.org/10.1016/j.celrep.2017.08.009>.
- Levin, Michael G., Tetsushi Nakao, Seyedeh M. Zekavat, et al. 2022. "Genetics of Smoking and Risk of Clonal Hematopoiesis." *Scientific Reports* 12 (1): 7248. <https://doi.org/10.1038/s41598-022-09604-z>.
- Li, Yuan-hui, Yu Zhang, Gang Pan, Li-xin Xiang, Ding-cun Luo, and Jian-zhong Shao. 2022. "Occurrences and Functions of Ly6Chi and Ly6Clo Macrophages in Health and Disease." *Frontiers in Immunology* 13 (May): 901672. <https://doi.org/10.3389/fimmu.2022.901672>.
- Lugade, Amit A, Paul N Bogner, Thomas H Thatcher, Patricia J Sime, Richard P Phipps, and Yasmin Thanavala. 2014. "Cigarette Smoke Exposure Exacerbates Lung Inflammation and Compromises Immunity to Bacterial Infection." *The Journal of Immunology* 192 (11): 5226–35. <https://doi.org/10.4049/jimmunol.1302584>.
- Mahmoudzadeh, leila, Seyyed Meysam Abtahi Froushani, Marjan Ajami, and Maryam Mahmoudzadeh. 2023. "Effect of Nicotine on Immune System Function." *Advanced Pharmaceutical Bulletin* 13 (1): 69–78. <https://doi.org/10.34172/apb.2023.008>.
- Marnell, Christopher S., Alexander Bick, and Pradeep Natarajan. 2021. "Clonal Hematopoiesis of Indeterminate Potential (CHIP): Linking Somatic Mutations, Hematopoiesis, Chronic Inflammation and Cardiovascular Disease." *Journal of Molecular and Cellular Cardiology* 161 (December): 98–105. <https://doi.org/10.1016/j.jmcc.2021.07.004>.
- Marques, Patrice, Laura Piqueras, and Maria-Jesus Sanz. 2021. "An Updated Overview of E-Cigarette Impact on Human Health." *Respiratory Research* 22 (1): 151. <https://doi.org/10.1186/s12931-021-01737-5>.

- Marston, Nicholas A., James P. Pirruccello, Giorgio E. M. Melloni, et al. 2024. "Clonal Hematopoiesis, Cardiovascular Events and Treatment Benefit in 63,700 Individuals from Five TIMI Randomized Trials." *Nature Medicine*, August 6, 1–7. <https://doi.org/10.1038/s41591-024-03188-z>.
- Masters, Andrew, and Paul Harrison. 2014. "Platelet Counting with the BD Accuri™ C6 Flow Cytometer." *Platelets* 25 (3): 175–80. <https://doi.org/10.3109/09537104.2013.801947>.
- McGrath, Kathleen E., Jenna M. Frame, and James Palis. 2015. "Early Hematopoiesis and Macrophage Development." *Seminars in Immunology*, Diversity of monocytes and macrophages, vol. 27 (6): 379–87. <https://doi.org/10.1016/j.smim.2016.03.013>.
- Mehta, Sakshi, and Veena Dhawan. 2020. "Exposure of Cigarette Smoke Condensate Activates NLRP3 Inflammasome in THP-1 Cells in a Stage-Specific Manner: An Underlying Role of Innate Immunity in Atherosclerosis." *Cellular Signalling* 72 (August): 109645. <https://doi.org/10.1016/j.cellsig.2020.109645>.
- Miller, Peter G., Dandi Qiao, Joselyn Rojas-Quintero, et al. 2022. "Association of Clonal Hematopoiesis with Chronic Obstructive Pulmonary Disease." *Blood* 139 (3): 357–68. <https://doi.org/10.1182/blood.2021013531>.
- Moran-Crusio, Kelly, Linsey Reavie, Alan Shih, et al. 2011. "Tet2 Loss Leads to Increased Hematopoietic Stem Cell Self-Renewal and Myeloid Transformation." *Cancer Cell* 20 (1): 11–24. <https://doi.org/10.1016/j.ccr.2011.06.001>.
- Ouyang, Yanli, Nisha Virasch, Ping Hao, et al. 2000. "Suppression of Human IL-1 β , IL-2, IFN- γ , and TNF- α Production by Cigarette Smoke Extracts." *Journal of Allergy and Clinical Immunology* 106 (2): 280–87. <https://doi.org/10.1067/mai.2000.107751>.
- Ożańska, Agnieszka, Donata Szymczak, and Justyna Rybka. 2020. "Pattern of Human Monocyte Subpopulations in Health and Disease." *Scandinavian Journal of Immunology* 92 (1): e12883. <https://doi.org/10.1111/sji.12883>.
- Patel, Amit A., Yan Zhang, James N. Fullerton, et al. 2017. "The Fate and Lifespan of Human Monocyte Subsets in Steady State and Systemic Inflammation." *Journal of Experimental Medicine* 214 (7): 1913–23. <https://doi.org/10.1084/jem.20170355>.
- Pietras, Eric M. 2017. "Inflammation: A Key Regulator of Hematopoietic Stem Cell Fate in Health and Disease." *Blood* 130 (15): 1693–98. <https://doi.org/10.1182/blood-2017-06-780882>.
- Ramanathan, Gajalakshmi, Jane H. Chen, Nitya Mehrotra, et al. 2023. "Cigarette Smoke Stimulates Clonal Expansion of Jak2V617F and Tet2-/- Cells." *Frontiers in Oncology* 13. <https://www.frontiersin.org/articles/10.3389/fonc.2023.1210528>.
- Ramanathan, Gajalakshmi, Brianna Craver-Hoover, Rebecca J. Arechavala, et al. 2020. "E-Cigarette Exposure Decreases Bone Marrow Hematopoietic Progenitor Cells." *Cancers* 12 (8): 2292. <https://doi.org/10.3390/cancers12082292>.

- Robinson, Amy, Claudia Z. Han, Christopher K. Glass, and Jeffrey W. Pollard. 2021. “Monocyte Regulation in Homeostasis and Malignancy.” *Trends in Immunology* 42 (2): 104–19. <https://doi.org/10.1016/j.it.2020.12.001>.
- Rossi, Derrick J., David Bryder, and Irving L. Weissman. 2007. “Hematopoietic Stem Cell Aging: Mechanism and Consequence.” *Experimental Gerontology* 42 (5): 385–90. <https://doi.org/10.1016/j.exger.2006.11.019>.
- Sarma, Nayan J., Akiko Takeda, and Nabeel R. Yaseen. 2010. “Colony Forming Cell (CFC) Assay for Human Hematopoietic Cells.” *Journal of Visualized Experiments: JoVE*, no. 46 (December): 2195. <https://doi.org/10.3791/2195>.
- Schuermans, Art, and Michael C. Honigberg. 2025. “Clonal Haematopoiesis in Cardiovascular Disease: Prognostic Role and Novel Therapeutic Target.” *Nature Reviews Cardiology*, April 2, 1–12. <https://doi.org/10.1038/s41569-025-01148-9>.
- Scott, Aaron, Sebastian T. Lugg, Kerrie Aldridge, et al. 2018. “Pro-Inflammatory Effects of e-Cigarette Vapour Condensate on Human Alveolar Macrophages.” *Smoking. Thorax* 73 (12): 1161–69. <https://doi.org/10.1136/thoraxjnl-2018-211663>.
- Seiler, Christopher L., J. ung Min Song, Delshanee Kotandeniya, et al. 2020. “Inhalation Exposure to Cigarette Smoke and Inflammatory Agents Induces Epigenetic Changes in the Lung.” *Scientific Reports* 10 (1): 11290. <https://doi.org/10.1038/s41598-020-67502-8>.
- Trzebanski, Sébastien, and Steffen Jung. 2020. “Plasticity of Monocyte Development and Monocyte Fates.” *Immunology Letters* 227 (November): 66–78. <https://doi.org/10.1016/j.imlet.2020.07.007>.
- Verma, Tanya, Srabani Sahu, Kith Pradhan, et al. 2025. “Cigarette Smoking Is Associated with Worse Prognosis in Myelodysplastic Syndromes and Hematopoietic Alterations in Murine Models.” *Leukemia & Lymphoma* 66 (10): 1964–68. <https://doi.org/10.1080/10428194.2025.2513675>.
- Ween, Miranda P., Jonathan J. Whittall, Rhys Hamon, Paul N. Reynolds, and Sandra J. Hodge. 2017. “Phagocytosis and Inflammation: Exploring the Effects of the Components of E-Cigarette Vapor on Macrophages.” *Physiological Reports* 5 (16): e13370. <https://doi.org/10.14814/phy2.13370>.
- Williams, Monique, Jun Li, and Prue Talbot. 2019. “Effects of Model, Method of Collection, and Topography on Chemical Elements and Metals in the Aerosol of Tank-Style Electronic Cigarettes.” *Scientific Reports* 9 (1): 13969. <https://doi.org/10.1038/s41598-019-50441-4>.
- Wohnhaas, Christian T., Kevin Baßler, Carolin K. Watson, et al. 2024. “Monocyte-Derived Alveolar Macrophages Are Key Drivers of Smoke-Induced Lung Inflammation and Tissue Remodeling.” *Frontiers in Immunology* 15. <https://doi.org/10.3389/fimmu.2024.1325090>.
- Wong, Kok Loon, June Jing-Yi Tai, Wing-Cheong Wong, et al. 2011. “Gene Expression Profiling Reveals the Defining Features of the Classical, Intermediate, and Nonclassical Human

- Monocyte Subsets.” *Blood* 118 (5): e16–31. <https://doi.org/10.1182/blood-2010-12-326355>.
- Wong, Kok Loon, Wei Hseun Yeap, June Jing Yi Tai, Siew Min Ong, Truong Minh Dang, and Siew Cheng Wong. 2012. “The Three Human Monocyte Subsets: Implications for Health and Disease.” *Immunologic Research* 53 (1): 41–57. <https://doi.org/10.1007/s12026-012-8297-3>.
- Xie, Zhuoer, and Amer M. Zeidan. 2023. “CHIPing Away the Progression Potential of CHIP: A New Reality in the Making.” *Blood Reviews* 58 (March): 101001. <https://doi.org/10.1016/j.blre.2022.101001>.
- Zarcone, Gianni, Marie Lenski, Thomas Martinez, et al. 2023. “Impact of Electronic Cigarettes, Heated Tobacco Products and Conventional Cigarettes on the Generation of Oxidative Stress and Genetic and Epigenetic Lesions in Human Bronchial Epithelial BEAS-2B Cells.” *Toxics* 11 (10): 10. <https://doi.org/10.3390/toxics11100847>.
- Zhao, Minghan, Yuting Han, Qi Yang, et al. 2023. “Evaluation of the Effects of E-Cigarette Aerosol Extracts and Tobacco Cigarette Smoke Extracts on RAW264.7 Cells.” *ACS Omega* 8 (32): 29336–45. <https://doi.org/10.1021/acsomega.3c02758>.

CHAPTER THREE

Electronic cigarette inhalation promotes the expansion of JAK2^{V617F} mutant hematopoietic stem cells

Jeanette YC Sullivan © 2025

ABSTRACT

Clonal hematopoiesis of indeterminate potential (CHIP) describes the presence of hematopoietic cells harboring driver mutations often found in myeloid malignancies. CHIP increases the risk for hematological disorders and cardiovascular disease. However, only a fraction of the individuals with CHIP eventually present with overt clonal hematopoiesis, and the mechanism behind their expansion into a malignant disease is not fully understood. The popularity of electronic cigarette (E-cig) usage has increased, but its health effects are not well-established. With their widespread availability and enticing flavors, they have also increased the number of nicotine consumers among teenagers and non-smokers. E-cigs have been associated with inflammation and oxidative stress, which can provide selective pressures for the outgrowth of CHIP mutant cells. The Jak2 gene is a driver mutation for the myeloproliferative neoplasm polycythemia vera (PV) and is commonly mutated in CHIP. We have previously shown that cigarette smoke promotes the survival of JAK2^{V617F} mutants in competitive mouse transplants. Here, we determined the impact of E-cig aerosol inhalation on the survival and expansion of Jak2-mutant hematopoietic cells by exposing bone marrow-transplanted chimeric JAK2^{V617F} mice to E-cig aerosols.

INTRODUCTION

In steady-state hematopoiesis, self-renewing hematopoietic stem cells (HSCs) constantly divide and differentiate to produce billions of blood and immune cells. Aberrant hematopoiesis and mutations can lead to blood disorders and fatal cancers. Myeloproliferative neoplasms (MPNs) are a heterogeneous group of rare, chronic blood cancers characterized by the excess proliferation of blood cells from the bone marrow. MPNs include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). PV, the most common MPN, causes an increase in red blood cells, which thickens the blood and dangerously increases the risk of thrombosis. In ET, excess platelets cause blood clots, increasing the chance of heart attacks or stroke. In PMF, the diseased bone marrow becomes inflamed and forms fibrotic scar tissue, impairing hematopoiesis. All MPNs are acquired genetic disorders, initiated by somatic mutations that arise after birth. While MPNs at their core are diseases of the blood and bone marrow, they also behave much like chronic inflammatory disorders (Soyfer and Fleischman 2023). Many of the most common MPN symptoms, such as pruritus (itching), night sweats, fatigue/weakness, rash, and fever, are also seen in inflammatory diseases. Although MPN symptoms can mostly be controlled with therapies, there is still no cure. Therefore, it is still of high clinical interest to understand the factors that promote the initiation and progression of MPNs.

Mutations in the Janus kinase 2 (JAK2) gene are among the most common mutations in MPN patients. The JAK2^{V617F} mutation is a conversion of valine (V) into phenylalanine (F) at the 617th amino acid of the gene. JAK2^{V617F} causes constitutive activation of JAK2. Constant activation of this JAK-STAT feedback loop can lead to increased pro-inflammatory gene expression (Geyer et al. 2015). MPN patients often have elevated levels of pro-inflammatory cytokines (Tefferi et al.

2011; Vaidya et al. 2012). These findings align with the fact that many MPN symptoms are inflammation-based. Accordingly, MPN research is particularly focused on controlling excess inflammation. Therefore, we seek to understand how inflammatory lifestyle stressors may exacerbate CHIP and associated immune responses.

One of the most prominent inflammatory lifestyle stressors is smoking behavior. Tobacco and nicotine use remain the leading preventable drivers of cancer risk, and both direct and secondhand exposure to combustible cigarettes or electronic nicotine devices perturbs immune function and hematopoiesis. We have previously demonstrated that *in vivo* exposure to traditional combustible cigarette smoke increased the proliferation of JAK2^{V617F} mutant cells (Ramanathan et al. 2023). In a separate study, we found that e-cigarette exposure suppresses the frequency of hematopoietic stem cells in the bone marrow (Ramanathan et al. 2020). Here, we seek to build upon these studies to assess the impact of e-cigarette exposure on the competition of JAK2^{V617F} cells. We hypothesize that e-cigarette exposure will promote the expansion of JAK2^{V617F} cells and increase the production of pro-inflammatory cytokines.

METHODS

Mice

All mice were approved under IACUC. All animal procedures were performed under the approval of the Institutional Animal Care and Use Committee at the University of California, Irvine. Wildtype C57BL/6J (Stock No. 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 8- to 10-weeks old and housed in specific pathogen-free facilities on a 12-hour light/dark cycle. Euthanasia was performed using an overdose of isoflurane, and euthanasia was confirmed by cervical dislocation. Where indicated, complete blood counts were obtained using the automated cell counter machine (ABCVet Hemalyzer, scilVet).

Competitive bone marrow transplantation

Fresh bone marrow was harvested from euthanized adult mice. Bone marrow was extracted by flushing the femurs and tibias with 10 mL of staining buffer using a syringe through a 45 μ m mesh strainer. The collected marrow was subjected to red blood cell lysis using ACK buffer on ice for 20 minutes, followed by washing and centrifugation. The resulting cell pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (D10). A small aliquot of the suspension was analyzed by flow cytometry to determine the total bone marrow cell count. Recipient mice were lethally irradiated with a single dose of 8Gy and transplanted retro-orbitally with approximately 1.2×10^6 whole bone marrow cells.

Chimerism flow cytometry

Peripheral blood was drawn using a bevel from the saphenous vein, and 20 μ L was collected into diluted EDTA. PB was treated with ACK buffer on ice for 20 minutes to lyse red blood cells, then washed and centrifugated. The cell pellet was washed with staining buffer (MilliQ water with FBS and PBS).

E-cigarette exposure

Exposures were performed at the Air Pollution Health Effects Laboratory at the University of California, Irvine. Exposures occurred 2 hours per day, 4 days/week, for 8 weeks. Between exposures, the mice were housed 4 per cage in an atmosphere-controlled room on a 12-hr light/dark cycle in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal housing facility at the University of California, Irvine vivarium. Aerosols were generated using a 2-s puff with a 60-s interval between puffs (35 mL puff volume) based on the ISO standard cigarette puff protocol (ISO 3308:2012) using a custom-built smoking system. This system uses a peristaltic pump to draw in aerosolized vapor from a Nichrome 70-watt E-cigarette. The e-liquid used was made by mixing equal volumes of propylene glycol (Millipore, Burlington, MA) and vegetable glycerin (Fisher Chemical, Hampton, NH) with 1.5% of nicotine (ThermoFisher, Waltham, MA). Control mice were exposed to air purified over potassium permanganate-impregnated alumina beads, activated carbon, and high-efficiency particulate air (HEPA) filters, concurrently with the combustion cigarette or e-cigarette exposures.

Bone marrow-derived macrophages (BMDMs)

Bone marrow was extracted by flushing the femurs and tibias with 10 mL of staining buffer using a syringe through a 45 μ m mesh strainer. The collected marrow was subjected to red blood cell lysis using ACK buffer on ice for 20 minutes, followed by washing and centrifugation. The resulting cell pellet was resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (D10). A small aliquot of the suspension was analyzed by flow cytometry to determine the total bone marrow cell count. To generate bone marrow-derived macrophages (BMDMs), 20 million bone marrow cells were resuspended in 10 mL of D10 supplemented with 30% L929 cell-conditioned media. Cells were seeded in non-tissue culture-treated 10 mm Petri dishes and incubated at 37°C in a 5% CO₂ atmosphere. After 3 days, each dish was supplemented with an additional 3 mL of fresh D10 + 30% L929 media. On day 5, media in each dish was replaced entirely with 10mL fresh D10 + 30% L929 media. After 7 days, the supernatant was discarded, and the macrophages were incubated with 1 mL of trypsin for 5 minutes. Macrophages were detached by scraping gently and collecting into D10.

Platelet P-selectin flow cytometry

50 μ l of washed blood was added to FACS tubes containing antibody mix of CD41-APC (MWReg30, ThermoFisher Scientific), P-selectin FITC (RB40.34, BD Biosciences), 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 using a BD Accuri C6 Plus.

ELISA cytokine detection

Concentrations of TNF- α and IL-6 were determined in conditioned media supernatant from cells incubated with 10ng/mL lipopolysaccharide (LPS, Sigma). Samples were frozen at -80oC until analysis. Commercial enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF- α (88-7324-88, Invitrogen/ThermoFisher) and mouse IL-6 (88-7325-23, Invitrogen/ThermoFisher) and used in accordance with the manufacturer's instructions.

Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM). GraphPad Prism (GraphPad Software, San Diego, CA) was used for all statistical analyses. Differences between mean values within groups were determined using ANOVA or unpaired t-tests where indicated.

RESULTS

E-cigarette exposure promotes the expansion of JAK2^{V617F} mutant cells

To investigate how e-cigarette use impacts the proliferation of JAK2^{V617F} mutant cells, we performed a competitive bone marrow transplant with bone marrow (BM) cells from wild-type (WT) C57BL/6J mice (CD45.1) and JAK2^{V617F} mice (CD45.2) into lethally irradiated WT recipients (CD45.1/2) (Figure 1A). As a WT control, we also performed simultaneous transplants with CD45.1 WT and CD45.2 WT into CD45.1/2 WT recipients (Figure 1A). One month after transplant, half of each transplant was exposed to nose-only inhalation of electronic cigarette aerosol (E-cig) for 2 hours per day, 4 days a week, for 6 months (Figure 1A). The other halves of each transplant were fitted into the nose cone and exposed to clean-room air during e-cigarette exposure. Our custom nose-only exposure apparatus limits uncontrolled dermal absorption that can result from whole-body smoke exposures (Figure 1B).

This study allows comparison of four distinct experimental transplant cohorts: e-cigarette-exposed WT: WT mice, air-exposed WT:WT mice, air-exposed WT: JAK2^{V617F} mice, and e-cigarette-exposed WT: JAK2^{V617F} mice (Figure 2A). We used flow cytometry to stain peripheral blood for CD45 chimerism to determine the relative proliferation of JAK2^{V617F} cells compared to WT CD45.1 competitor cells, with CD45.2 being either a JAK2^{V617F} or WT competitor. In competitive bone marrow transplants in a 1:1 ratio, this mouse model of JAK2^{V617F} does not gain a selective advantage over WT cells. Instead, we observe declining levels of JAK2^{V617F} cells in the peripheral blood over time. We observed that the input frequency of 50% JAK2^{V617F} cells resulted in ~30% JAK2^{V617F} frequency at one month post-transplant (Figure 2A). Six months of

e-cigarette exposure significantly increased the frequency of JAK2V617F cells in the peripheral blood in comparison to the air exposed mice.

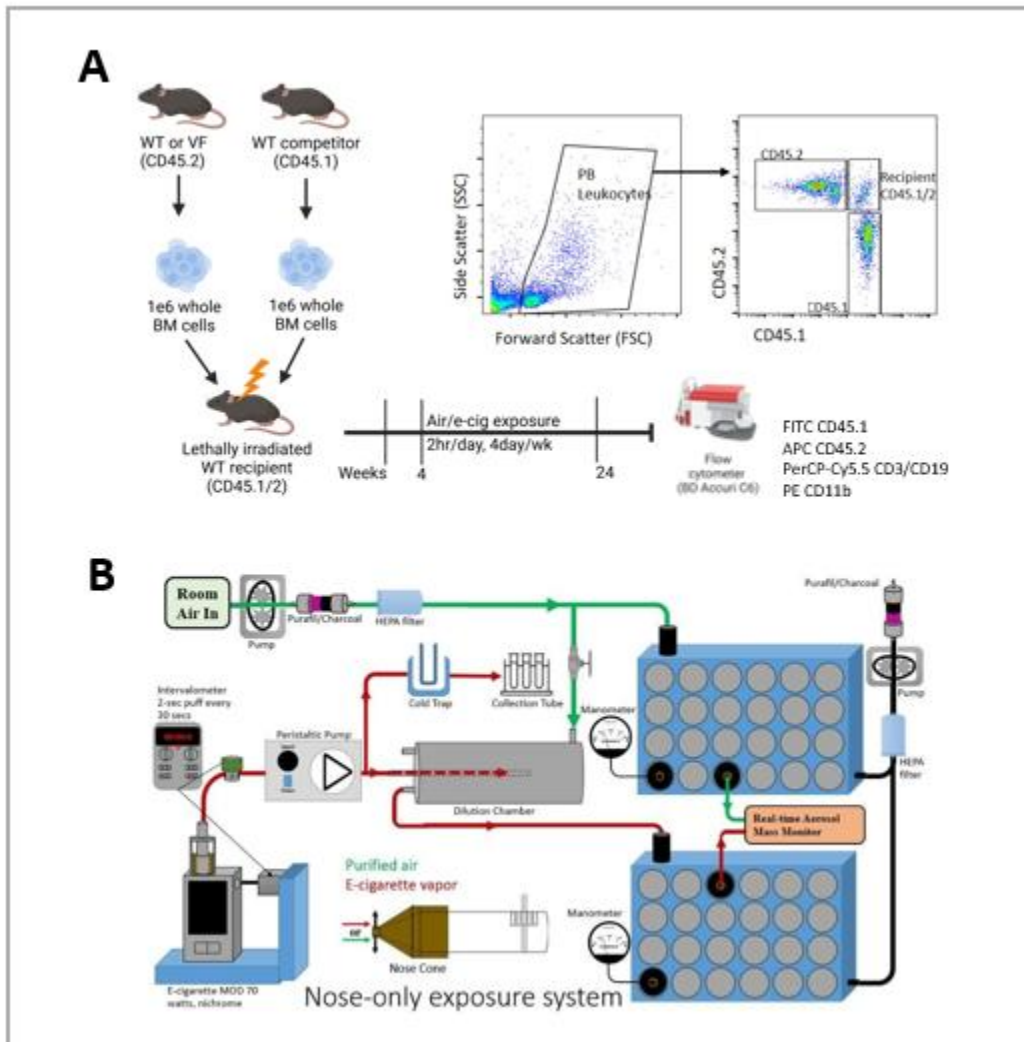


Figure 1. Experimental setup of WT:JAK2V617F and WT:WT transplants for in vivo e-cigarette exposure. (A) Whole BM was freshly isolated from JAK2^{V617F} (VF) and WT mice. Two million whole BM cells, 1e6 from the WT (CD45.1) donor and 1e6 from the WT (CD45.2) or VF (CD45.2) donor, were transplanted into lethally irradiated CD45.1/2 WT recipients. Mice were bled by the saphenous vein to determine peripheral blood chimerism monthly. Mice were exposed to e-cigarette aerosol or clean room air through a nose-only exposure system depicted in (B) for two hours/day, four days/week.

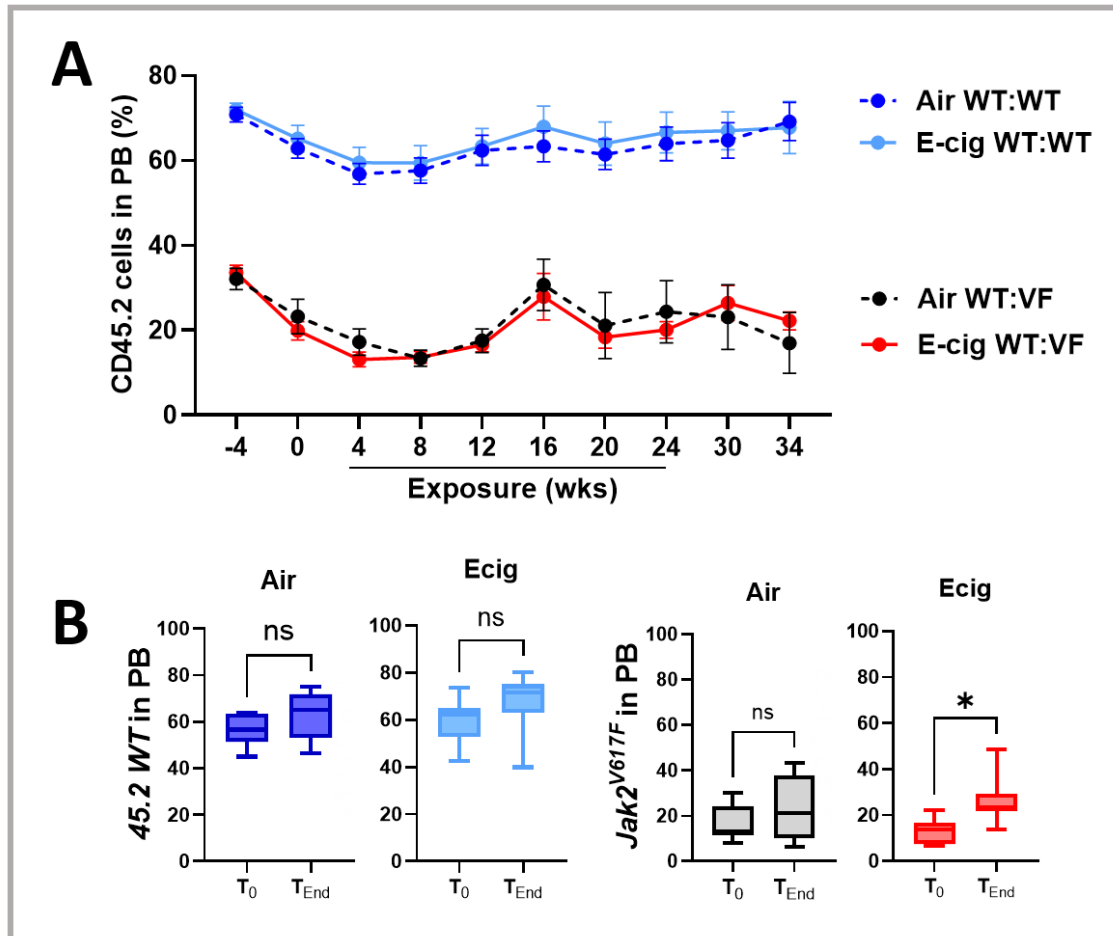


Figure 3. Mice exposed to E-cig aerosols demonstrated significant expansion of JAK2V617F cells at the end of the experiment compared to air-exposed controls. (A) Contribution of CD45.2 (WT or JAK2V617F) to peripheral blood as a percentage of total cells. Transplanted at week n= -4; exposure from weeks 4-24. (B) contribution of CD45.2 (WT or JAK2V617F) to PB as a percentage of all leukocytes. Time point 0 (T₀) is the beginning of exposure (week 8) and T_{End} is termination of the experiment. Values represent mean+SEM. Significance determined by Wilcoxon paired test, *p<0.05

E-cigarette exposure impairs inflammatory cytokine production

To further explore the impact of e-cigarette exposure on cytokine production, we compared LPS-induced inflammatory cytokine production in bone marrow-derived macrophages (Figure 4). The

secretion of the pro-inflammatory cytokines, TNF α and IL-6, was quantified by ELISA. We did not observe quantifiable TNF- α or IL-6 secretion from steady-state, unstimulated BMDMs (results not shown). To mimic an immune assault, BMDMs from exposed mice were stimulated with LPS. TNF- α production did not differ among groups (Figure 3A). WT: WT BMDMs from the E-cig cohort produced more IL-6 than the WT: WT BMDMs from the air cohort (Figure 3B). However, E-cig-exposed WT: JAK2^{V617F} BMDMs produced less LPS-induced TNF α and IL-6 than the E-cig-exposed WT: WT BMDMs (Figure 3). Notably, in the JAK2^{V617F} transplant, the E-cigarette-exposed JAK2^{V617F} mice had significantly impaired IL-6 production than the air-exposed JAK2^{V617F} mice (Figure 3). These results suggest that JAK2^{V617F} mutations increase susceptibility to e-cigarette-induced immune dysfunction.

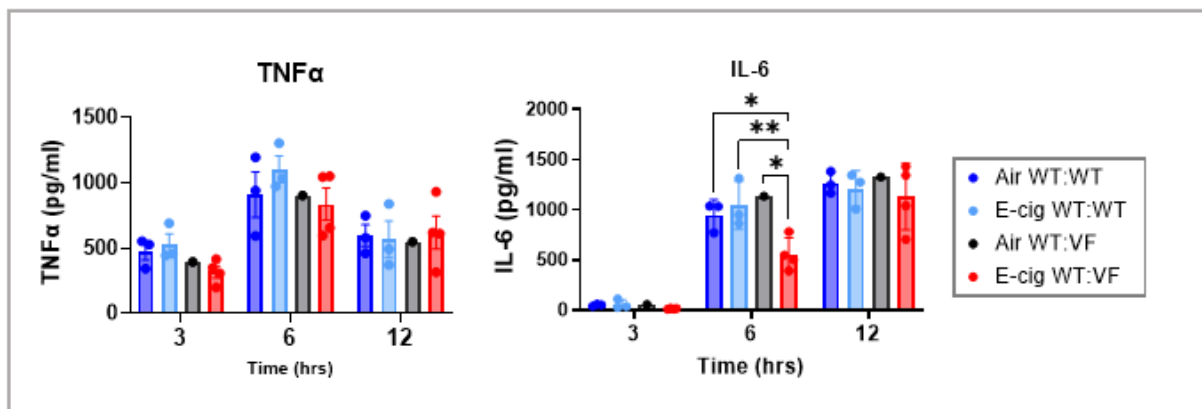


Figure 4. Cytokine secretion in bone marrow-derived macrophages from mice exposed to air or e-cigarette aerosol. Bone marrow from each mouse was harvested and differentiated into macrophages for one week. BMDMs were washed and incubated with 10ng/mL LPS for 3-12 hours. Supernatant was collected at the indicated time point and frozen at -80 °C until analyzed by ELISA for (A) TNF- α and (B) IL-6. Values represent means $\pm$ SEM.

E-cigarette exposure increases P-selectin expression on platelets in JAK2^{V617F} mice

The most prominent clinical concern for JAK2^{V617F} MPN patients is the risk of thrombosis. During thrombosis, a blood clot, or thrombus, forms from aggregates of platelets and leukocytes that clog blood vessels and impair blood flow (Engelmann and Massberg 2013). To assess whether e-cigarette inhalation affects platelet function, we measured P-selectin expression on circulating platelets. P-selectin, or CD62p, is expressed on activated platelets and is key in the recruitment of monocytes and other inflammatory immune cells. (Nocella et al. 2018). E-cig-exposed WT: JAK2^{V617F} BMDMs had significantly increased P-selectin expression compared to the air-exposed WT: JAK2^{V617F} BMDMs, as well as both the air- and e-cig-exposed WT BMDMs (Figure 4).

Post-e-cigarette exposure bone marrow and spleen JAK2^{V617F} chimerism

To determine the impact of e-cigarette exposure on HSCs, we analyzed JAK2^{V617F} chimerism in the bone marrow progenitor cells. We also analyzed the spleen cells for extramedullary hematopoiesis. In healthy mice, the spleen retains a small reservoir of hematopoietic stem cells but does not contribute considerably to hematopoiesis overall. However, a characteristic of MPN is splenomegaly and increased HSC activity in the spleen. Contrary to the peripheral blood, the E-cigarette-exposed mice did not have significantly higher JAK2^{V617F} frequency than the air-exposed mice (Figure 5).

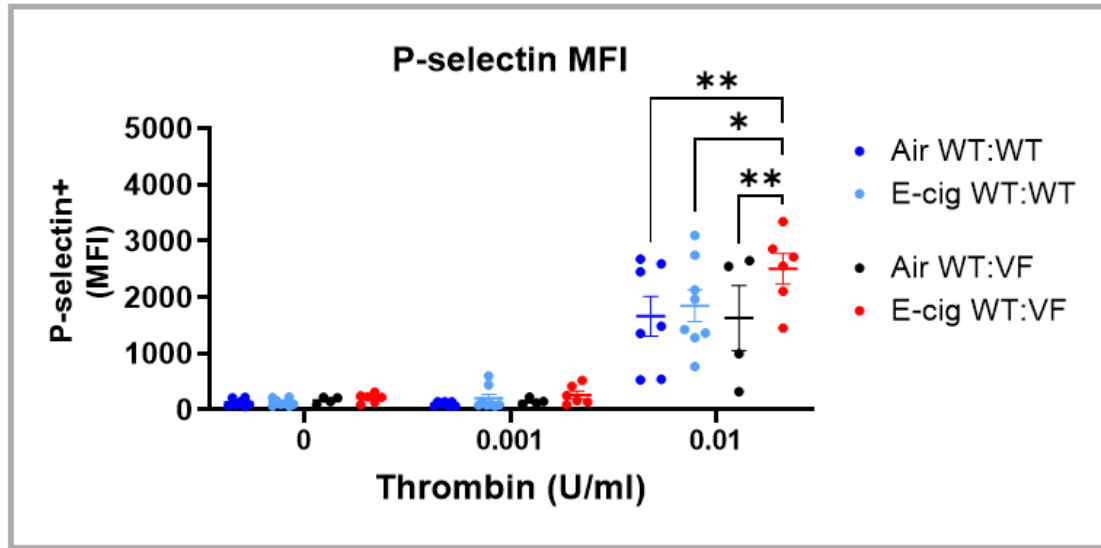


Figure 6. P-selectin mean fluorescence intensity (MFI) following thrombin-stimulation on platelets. Whole blood was activated with thrombin and incubated with antibodies against CD41 and P-selectin. Mean fluorescence intensity was measured using flow cytometry. Significance determined by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$. Values represent means $\pm$ SEM.

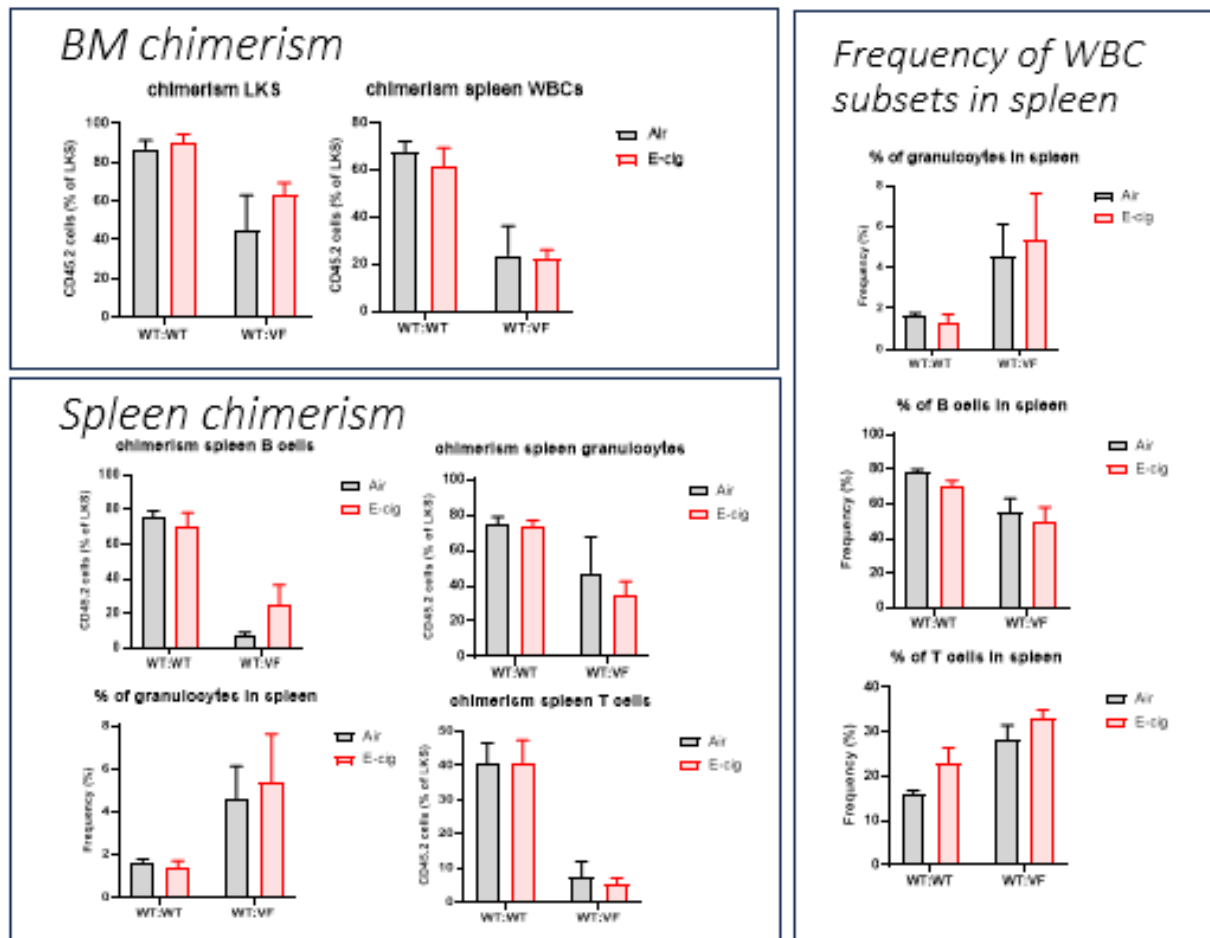


Figure 8. Terminal bone marrow and spleen chimerism after e-cigarette exposure. Transplanted mice were sacrificed and bone marrow and spleen harvested. Roughly half of the spleen was crushed and cells harvested through a 0.22uM filter. Bone marrow was collected by flushing femurs and tibias. BM and spleen cells were washed and stained for chimerism and cell surface marker expression to differentiate cell types. Significance analyzed by one-way ANOVA.

DISCUSSION

In this study, we demonstrated that e-cigarette exposure can preferentially expand JAK2^{V617F} cells over wild-type counterparts. This suggests that using e-cigarettes may not be safe for JAK2^{V617F} patients and may be risky for those harboring underlying CHIP mutations.

Christensen et al reported that in a study of MPN patients, current and former smokers had higher overall symptom burden and significantly lower quality of life (Christensen et al. 2021).

In human patients, the JAK2^{V617F} mutation burden is positively correlated with MPN development and progression (Nielsen et al. 2014). Here, we have identified that smoking behavior itself, including the “safe” alternative of e-cigarettes, could be increasing the JAK2^{V617F} mutation burden.

We also observed attenuation of inflammatory responses in bone marrow-derived macrophages. IL-6, or Interleukin-6, plays a key role in many physiological processes, including inflammation, hematopoiesis, bone metabolism, and more (Pedersen et al. 2020). E-cigarette-exposed JAK2^{V617F} BMDMs produced significantly less IL-6, suggesting that macrophages are more resistant to LPS-mediated inflammation after E-cigarette exposure. This suggests that E-cigarettes sensitize WT cells to inflammation more than JAK2^{V617F} mutant cells. This finding is contrary to our hypothesis, as the literature suggests a pro-inflammatory effect of both traditional and electronic cigarettes. However, a limitation of this study is that we did not quantify circulating cytokine levels, which would more likely show a general increase in inflammatory cytokines after cigarette exposure. Here, we instead focus on LPS-induced inflammatory cytokine production, which mimics the ability of cells to respond to an immune assault, rather than their resting state. LPS-induced impairments imply increased susceptibility to infection, which has also been observed in chronic smokers.

P-selectin, or CD62p, is expressed on activated platelets and is key in the recruitment of monocytes and other inflammatory immune cells. Nocella et al demonstrated that both tobacco- and e-cigarette use increases platelet activation, and Loke et al observed aggravated platelet aggregation in smokers (Nocella et al. 2018; Loke et al. 2014). Monocytes and neutrophils in circulation interact with platelets to form leukocyte-platelet aggregates (Shen and Wu 2022). We demonstrated that e-cigarette exposure significantly increased the expression of P-selectin on platelets, implying that the JAK2^{V617F} mutation increases e-cigarette-induced platelet activation.

Future studies may focus on further exploring the mechanisms by which both cigarette smoke and e-cigarette vape inhalation might promote the expansion of JAK2^{V617F} cells. One theory is that e-cigarettes induce a chronic inflammatory state in the body, causing hematopoietic stem cell stress that allows the mutant cells to proliferate. Future investigations could include more detailed analysis of peripheral blood composition and the activation states of monocytes and macrophages or further investigate the dual effects of JAK2^{V617F} mutations and e-cigarette exposure on platelet P-selectin expression. Additionally, the e-cigarette aerosol used in this study is an unflavored mixture of PG/VG and nicotine. Studies suggest that different e-cigarette flavors can have distinct effects on immune cell function in the lungs and beyond. Incorporating multiple flavors into the exposures would be of interest from a regulatory perspective.

Understanding the role of environmental factors such as combustible cigarette smoke and E-cigarette products on normal and mutant HSCs may be valuable in adequately addressing the clinical consequences of CHIP.

REFERENCES

- Christensen, Sarah F., Robyn M. Scherber, Gina L. Mazza, et al. 2021. "Tobacco Use in the Myeloproliferative Neoplasms: Symptom Burden, Patient Opinions, and Care." *BMC Cancer* 21 (1): 691. <https://doi.org/10.1186/s12885-021-08439-7>.
- Engelmann, Bernd, and Steffen Massberg. 2013. "Thrombosis as an Intravascular Effector of Innate Immunity." *Nature Reviews. Immunology* 13 (1): 34–45. <https://doi.org/10.1038/nri3345>.
- Geyer, Holly L., Amylou C. Dueck, Robyn M. Scherber, and Ruben A. Mesa. 2015. "Impact of Inflammation on Myeloproliferative Neoplasm Symptom Development." *Mediators of Inflammation* 2015 (1): 284706. <https://doi.org/10.1155/2015/284706>.
- Loke, Wai Mun, Karen Lam Mok Sing, Chung-Yung J. Lee, et al. 2014. "Cyclooxygenase-1 Mediated Platelet Reactivity in Young Male Smokers." *Clinical and Applied Thrombosis/Hemostasis* 20 (4): 371–77. <https://doi.org/10.1177/1076029612466284>.
- Nielsen, Camilla, Stig E. Bojesen, Børge G. Nordestgaard, Klaus F. Kofoed, and Henrik S. Birgens. 2014. "JAK2V617F Somatic Mutation in the General Population: Myeloproliferative Neoplasm Development and Progression Rate." *Haematologica* 99 (9): 9. <https://doi.org/10.3324/haematol.2014.107631>.
- Nocella, Cristina, Giuseppe Biondi-Zoccai, Sebastiano Sciarretta, et al. 2018. "Impact of Tobacco Versus Electronic Cigarette Smoking on Platelet Function." *The American Journal of Cardiology* 122 (9): 1477–81. <https://doi.org/10.1016/j.amjcard.2018.07.029>.
- Pedersen, Kasper Mønsted, Yunus Çolak, Christina Ellervik, Hans Carl Hasselbalch, Stig Egil Bojesen, and Børge Grønne Nordestgaard. 2020. "Loss-of-Function Polymorphism in IL6R Reduces Risk of JAK2V617F Somatic Mutation and Myeloproliferative Neoplasm: A Mendelian Randomization Study." *eClinicalMedicine* 21 (April). <https://doi.org/10.1016/j.eclinm.2020.100280>.
- Ramanathan, Gajalakshmi, Jane H. Chen, Nitya Mehrotra, et al. 2023. "Cigarette Smoke Stimulates Clonal Expansion of Jak2V617F and Tet2-/- Cells." *Frontiers in Oncology* 13. <https://www.frontiersin.org/articles/10.3389/fonc.2023.1210528>.
- Ramanathan, Gajalakshmi, Brianna Craver-Hoover, Rebecca J. Arechavala, et al. 2020. "E-Cigarette Exposure Decreases Bone Marrow Hematopoietic Progenitor Cells." *Cancers* 12 (8): 2292. <https://doi.org/10.3390/cancers12082292>.
- Shen, Chih-Lung, and Yi-Feng Wu. 2022. "Flow Cytometry for Evaluating Platelet Immunophenotyping and Function in Patients with Thrombocytopenia." *Tzu-Chi Medical Journal* 34 (4): 381–87. https://doi.org/10.4103/tcmj.tcmj_117_22.
- Soyfer, Eli M., and Angela G. Fleischman. 2023. "Myeloproliferative Neoplasms – Blurring the Lines between Cancer and Chronic Inflammatory Disorder." *Frontiers in Oncology* 13 (June). <https://doi.org/10.3389/fonc.2023.1208089>.
- Tefferi, Ayalew, Rakhee Vaidya, Domenica Caramazza, Christy Finke, Terra Lasho, and Animesh Pardanani. 2011. "Circulating Interleukin (IL)-8, IL-2R, IL-12, and IL-15 Levels Are Independently Prognostic in Primary Myelofibrosis: A Comprehensive Cytokine Profiling Study." *Journal of Clinical Oncology* 29 (10): 1356–63. <https://doi.org/10.1200/JCO.2010.32.9490>.

Vaidya, Rakhee, Naseema Gangat, Thitina Jimma, et al. 2012. "Plasma Cytokines in Polycythemia Vera: Phenotypic Correlates, Prognostic Relevance, and Comparison with Myelofibrosis." *American Journal of Hematology* 87 (11): 1003–5. <https://doi.org/10.1002/ajh.23295>.

CHAPTER FOUR

SUMMARY & CONCLUSIONS

In this dissertation, we investigated the impact of smoking both traditional tobacco cigarettes and modern electronic cigarettes on hematopoiesis stem cell function and immune responses in multiple models of clonal hematopoiesis. Given the high rate of smoking, alarming rise in e-cigarette use, and literature that increasingly validates the clinical significance of clonal hematopoiesis, it is imperative to characterize the health risks of these products. The overarching goal of this project was to further understand how an inflammatory lifestyle stressor, such as smoking, contributes to aberrant hematopoiesis in wild-type normal stem cells, Tet2-deficient cells, and JAK2^{V617F} mutant cells.

In Chapter Two, we generated extracts from tobacco research cigarettes and a commercially available electronic cigarette. Using extracts opens up new avenues of *in vitro* cell culture techniques into which cigarette smoke and e-cigarette aerosol can be easily incorporated. The CSE and EVE allowed us to directly investigate their impact on hematopoietic stem cells. To our knowledge, this is the first study that tracks the differentiation dynamics of HSCs exposed to cigarette and e-cigarette extracts in an *in vivo* bone marrow transplant. Mice transplanted with EVE-exposed BM had increased frequencies of Tet2-deficient cells in the bone marrow and peripheral blood several months post-transplant. Additionally, mice transplanted with both EVE- and/or CSE-exposed BM had deranged hematopoiesis at over 9 months post-exposure, demonstrating that smoke products have enduring impacts on HSCs. This novel technique not only demonstrates that cigarette and e-cigarette extracts can directly modulate HSC function but also

shows that freshly harvested bone marrow can be incubated overnight with low concentrations of these formulations and subsequently transplanted successfully. An advantage of these techniques is that they allow establishing a smoke exposure model without the logistical challenges of whole-body or nose-only exposure setups.

In these studies, we erred on the side of caution and used very low concentrations of CSE and EVE, ranging from 0.5% to 2%. Our goal was to elucidate the effects of exposure on HSCs without significant cell death. Given the success of the long-term hematopoietic engraftment and survival in the mice transplanted with EVE- and CSE-exposed BM, future studies could feasibly include higher concentrations of extracts or different e-liquid sources. Additionally, our competitive transplant began with a WT: Tet2^{-/-} ratio of 93%: 7%. Tet2-deficient cells have a strong competitive advantage over WT cells, so even a minimal input frequency will still allow them to proliferate rapidly. This is indeed what we observed, even more so than expected; in the “control” group, the Tet2-deficient cells expanded to a frequency of ~70% peripheral blood leukocytes. Subsequent studies could lower the input Tet2^{-/-} frequency even more to more effectively mimic a low-level CHIP state.

As described in Chapter One, the studies of how cigarette and e-cigarette smoking are contradictory. To address this discrepancy, we examined the effects of CSE and EVE on LPS-induced inflammatory responses across multiple macrophage models. Surprisingly, we observed a suppression of TNF- α secretion in response to CSE. We demonstrated this dose-dependent effect of CSE on human and mouse cell lines, on freshly isolated bone marrow-derived macrophages from wild-type and Tet2^{-/-} mice, and in human PBMCs. This finding encapsulates one of the central debates in nicotine research: that there is evidence for both immunosuppression and excessive inflammation. These seemingly opposite situations can coexist, supporting the more complex

theory that chronic inflammation impairs an acute immune response. We theorize that chronic nicotine use over time increases overall levels of circulating inflammatory cytokines but inhibits the ability to respond to an immune stimulus, hence serving both pro- and anti-inflammatory roles. This finding from Chapter One aligns with our results in Chapter Two, where we observed that e-cigarette-exposed JAK2^{V617F} BMDMs secreted less IL-6 in response to LPS stimulation than both air-exposed WT and JAK2^{V617F} BMDMs. In Chapter Three, we further demonstrated that e-cigarette exposure promotes the expansion of JAK2^{V617F} cells in the peripheral blood. Altogether, these results suggest that e-cigarettes have mutagenic potential for CHIP mutations.

A question that remains from these studies is whether the negative hematopoietic impacts of both combustible and electronic cigarette smoking are permanent or transient. A patient cannot rewrite having a history of smoking, but they can change their behavior in the future. Public awareness of the negative long-term health impacts of chronic inflammation has risen, prompting increased interest in anti-inflammatory supplements. In Chapter Two, we performed long-term *in vivo* exposures to cigarette smoke and e-cigarette aerosol sequentially. We found that both types of exposure impair the hematopoietic reconstitution ability, represented by skewed myeloid differentiation in a secondary transplant. Myeloid bias is a hallmark of HSC aging (Rossi et al. 2007). To further study the impact of age on transplantation with BM from smoke-exposed mice, we tracked the longevity of the secondary transplant for just over a year. Interestingly, the myeloid bias emerged after 6 months of age, suggesting that smoke and e-cigarette exposure imprint long-lasting changes on stem cells that persist through serial transplantation and functionally resemble accelerated HSC aging. The impacts of smoke and e-cigarette exposure carried over to a secondary transplant, demonstrating that smoking cessation does not completely return cells to a homeostatic

state. In a clinical context, this suggests the need for interventions that might undo smoking-related damage.

The dietary supplement N-acetylcysteine (NAC) has antioxidant and anti-inflammatory properties. NAC is a precursor to glutathione, a critical antioxidant that plays roles in immune function, reducing oxidative stress, regulating blood pressure, and more. NAC is promoted as a nicotine dependence treatment to prevent cravings and relapse (Schmaal et al. 2011; Machado et al. 2020). Administration of NAC may also be supplemented with glycine, purported to increase the efficacy of NAC (Kumar et al. 2021). The techniques used in this dissertation, such as competitive transplants with CSE- and EVE-exposed bone marrow and nose-only exposures, can be tested with NAC and/or glycine to determine if the antioxidant can reduce the impacts of smoking on HSCs.

Another unexplored aspect to consider is the further characterization of CHIP mutant cells regardless of their competitive ability against wild-type cells. Interestingly, individuals with CHIP have both an increased risk of COPD and increased disease severity (Miller et al. 2022). In fact, Miller et al. demonstrated that mice with hematopoietic Tet2 deficiency developed significantly more emphysema after 6 months of cigarette smoke exposure, notably without expansion of the Tet2^{-/-} cells. In Chapter Two, the cigarette smoke extract (CSE) did not significantly increase the frequency of Tet2-deficient cells in the peripheral blood. However, the CSE still skewed myeloid differentiation and increased the frequency of the inflammatory monocyte subset. The CSE could increase the malignancy of a Tet2-deficient HSC population without increasing its proliferative capacity. A deeper characterization of the impact of cigarette smoke on Tet2-deficient cells, both LPS-stimulated and non-stimulated, would provide further insight into its effects on hematopoiesis and other disease associations.

In these studies, we focused on Tet2-knockout cells and JAK2V617F mutant cells. However, there are several other CHIP mutations that are also correlated with smoking behavior. DNMT3A, the most frequently mutated CHIP gene, is DNA methyltransferase 3A, which regulates de novo CpG methylation (Tajiri et al. 2023; Mayle et al. 2015; Cobo, T. Tanaka, et al. 2022; Yu et al. 2023). Loss of function of DNMT3a favors HSC self-renewal over differentiation and predisposes cells to malignant transformation (Mayle et al. 2015; Jeong et al. 2018). Approximately 60% of acute myeloid leukemia (AML) patients have heterozygous mutations at arginine 882 (R882), causing disrupted methylation (Mayle et al. 2015). In humans, DNMT3A R882H is associated with worse overall leukemia outcomes and higher therapeutic resistance (Liao et al. 2022). Loss of function of DNMT3a or TET2 caused mitochondrial DNA degradation in human macrophages (Tajiri et al. 2023; Cobo, T. N. Tanaka, et al. 2022). *ASXL1*, *Additional sex combs-like 1*, is a key chromatin regulator. Mutations in *ASXL1* have been observed at levels of 10% in MPNs, 20% in MDS and AML, and 45% in chronic myelomonocytic leukemia (CMML) (Gao et al. 2022). In mice, *ASXL1* deletion leads to myeloid and erythroid dysplasia and reduced HSC self-renewal (Abdel-Wahab et al. 2013). ASXL1-knockout splenocytes have lower global levels of histone modifications H3k4me1, H3K4me3, and H3K27me3 (You et al. 2022). These CHIP mutations have also been correlated to smoking behavior, particularly ASXL1 (Levin et al. 2022; Dawoud et al. 2020; Kessler et al. 2022). Incorporation of other CHIP mutations into studies of smoking and hematopoiesis would further our understanding of these correlations.

Next to smoking, dietary choices also have a substantial impact on health and disease risk. Future studies might incorporate a second inflammatory stressor, such as the cumulative effects of both smoking and Western or high-fat diets. Traditional cigarette smoke consumption is linked to cancer, CVD, and CHIP. Atherosclerosis is an inflammatory disease, and one way that

hematopoietic stem cells may regulate atherosclerosis is through the macrophage (Hilgendorf et al. 2015; Wang et al. 2018; Tomas et al. 2021). In atherosclerosis, monocytes infiltrate the tissue and differentiate into lesional macrophages that uptake oxidized low-density lipoproteins (oxLDL) via scavenger receptors to become foam cells. These lipid-laden foam cells become clustered necrotic cores in atherosclerotic plaques in major arteries (Gui et al. 2022; Chen et al. 2020; Kim et al. 2020). Foam cell formation is governed by the sum effects of lipid uptake, lipid efflux, and cholesterol esterification (Gui et al. 2022). Chronically elevated blood lipid levels trigger lipoprotein deposits to form in vessel walls (Heyde et al. 2021). Circulating reactive oxygen species (ROS) generate oxLDL and trigger cells to increase adhesion molecule expression, prompting migration of monocytes and subsequent macrophage differentiation and foam cell formation (Alakhtar et al. 2023). Preliminary data from the Fleischman lab indicate that CSE increases the formation of foam cells by bone marrow-derived macrophages from wild-type mice. E-cigarettes have been shown to induce pro-atherogenic markers in hyperlipidemia-prone mice (Alakhtar et al. 2023). In atherosclerosis-prone mice, whole-body e-cigarette exposure induced accelerated atherosclerotic development and mitochondrial DNA damage (Li et al. 2021). An interesting experiment would be to study the combined effects of high-fat diets and nose-only cigarette exposure in transplanted mice to determine whether a double inflammatory assault would further promote the expansion of Tet2-knockout or JAK2^{V617F} mutant hematopoietic stem cells.

Additionally, we identified in Chapter Three that e-cigarette exposure increased P-selectin expression on platelets in JAK2^{V617F} mice. Along with the incorporation of atherosclerosis research ideas, of particular interest for JAK2^{V617F} mice would be to assess the increased risk of thrombosis after cigarette and e-cigarette exposure. Future studies could incorporate models of *in vivo* acute pulmonary thrombosis or measurement of platelet-leukocyte aggregates (Craver et al.

2020). High levels of platelet-leukocyte aggregates are correlated to increased thrombotic risk in MPN patients (Jensen et al. 2001). Agonists for the nicotinic acetylcholine receptors (nAChRs), which nicotine binds to, could also lend further insight into how nicotine regulates cell signaling.

The importance of including both male and female subjects is also a major topic in the toxicology field. In these studies, we did not identify any significant differences between male and female mice or male and female human subjects. However, research suggests that there are notable differences in male and female inflammatory responses (Giles et al. 2025). Sex and gender are crucial aspects of health, but these variables are often neglected, usually at the expense of female subjects. Halsted et al identified differences in male and female endothelial cell function and oxidative stress after e-cigarette use (Halstead et al. 2023). Increased incorporation of male vs female studies, especially in combination with atherosclerosis-related research questions, would be a valuable investigation.

Commercially available e-cigarette liquids are highly heterogeneous and differ in their ratios of propylene glycol (PG) and vegetable glycerin (VG), nicotine content, and flavor additives. There is still relatively little regulation on the additives and flavoring that are in these products. PG and VG are classified as “Generally Recognized As Safe” (GRAS) by the FDA. Evidence suggests that different e-cigarette flavors can have distinct effects on inflammation; one brand or flavor may induce significant oxidative stress, while another does not. If e-cigarette products are here to stay, toxicology guidelines can be utilized to optimize the safety of e-cigarettes. This research could be further adapted and expanded to promote extensive regulatory parameters. For example, one might establish a list of hazardous biomarkers that e-cigarette formulations must prove they do not induce. While this research does suggest mutagenic potential of e-cigarette products, we are unlikely to ever fully eliminate smoking behavior from the population. Therefore,

if there are ways to create potentially safer nicotine consumption alternatives, while not perfect, could still have an appreciable impact on public health.

In conclusion, this work has explored how traditional and electronic cigarettes contribute to the expansion of inflammatory CHIP mutations. We performed novel techniques combining toxicology with competitive bone marrow transplants to observe hematopoietic stem cell differentiation. The JAK2^{V617F} mutation drives MPN pathogenesis, and the frequency of JAK2V617F cells in peripheral blood increased after 6 months of e-cigarette use. E-cigarette extracts promoted proliferation of Tet2-deficient cells. Both cigarette and e-cigarette exposure, *in vivo* and *in vitro*, skewed myeloid differentiation and promoted an aging HSC phenotype. The mechanisms by which the specific components of e-cigarettes select for these CHIP mutations remain a subject of interest for future studies. Ultimately, we can conclude that inflammatory lifestyle stressors have a significant impact on the proliferation of mutant hematopoietic cells.

REFERENCES

- Abdel-Wahab, Omar, Jie Gao, Mazhar Adli, et al. 2013. "Deletion of Asxl1 Results in Myelodysplasia and Severe Developmental Defects in Vivo." *The Journal of Experimental Medicine* 210 (12): 2641–59. <https://doi.org/10.1084/jem.20131141>.
- Alakhtar, Bayan, Cynthia Guilbert, Nivetha Subramaniam, et al. 2023. "E-Cigarette Exposure Causes Early pro-Atherogenic Changes in an Inducible Murine Model of Atherosclerosis." *Frontiers in Toxicology* 5 (December): 1244596. <https://doi.org/10.3389/ftox.2023.1244596>.
- Chen, Wujun, Yudong Wu, Qi Lu, Shuai Wang, and Dongming Xing. 2020. "Endogenous ApoA-I Expression in Macrophages: A Potential Target for Protection against Atherosclerosis." *Clinica Chimica Acta* 505 (June): 55–59. <https://doi.org/10.1016/j.cca.2020.02.025>.
- Cobo, Isidoro, Tiffany Tanaka, Christopher K. Glass, and Calvin Yeang. 2022. "Clonal Hematopoiesis Driven by DNMT3A and TET2 Mutations: Role in Monocyte and Macrophage Biology and Atherosclerotic Cardiovascular Disease." *Current Opinion in Hematology* 29 (1): 1–7. <https://doi.org/10.1097/MOH.0000000000000688>.
- Cobo, Isidoro, Tiffany N. Tanaka, Kailash Chandra Mangalhara, et al. 2022. "DNA Methyltransferase 3 Alpha and TET Methylcytosine Dioxygenase 2 Restrains Mitochondrial DNA-Mediated Interferon Signaling in Macrophages." *Immunity* 55 (8): 1386–1401.e10. <https://doi.org/10.1016/j.immuni.2022.06.022>.
- Craver, Brianna M., Gajalakshmi Ramanathan, Summer Hoang, et al. 2020. "N-Acetylcysteine Inhibits Thrombosis in a Murine Model of Myeloproliferative Neoplasm." *Blood Advances* 4 (2): 312–21. <https://doi.org/10.1182/bloodadvances.2019000967>.
- Dawoud, Ahmed A. Z., William J. Tapper, and Nicholas C. P. Cross. 2020. "Clonal Myelopoiesis in the UK Biobank Cohort: ASXL1 Mutations Are Strongly Associated with Smoking." *Leukemia* 34 (10): 10. <https://doi.org/10.1038/s41375-020-0896-8>.
- Gao, Xin, Xiaona You, Nathalie Droin, et al. 2022. "Role of ASXL1 in Hematopoiesis and Myeloid Diseases." *Experimental Hematology* 115 (November): 14–19. <https://doi.org/10.1016/j.exphem.2022.09.003>.
- Giles, Braeden H, Nivetha Kamalavannan Subramaniam, Vincenza Caruana, Nikola Kukolj, Bernard Robaire, and Koren K Mann. 2025. "A Mixture of Organophosphate Esters Sex-Specifically Impacts Monocyte–Macrophages in Sprague-Dawley Rats." *Toxicological Sciences*, May 28, kfaf078. <https://doi.org/10.1093/toxsci/kfaf078>.
- Gui, Yuzhou, Hongchao Zheng, and Richard Y. Cao. 2022. "Foam Cells in Atherosclerosis: Novel Insights Into Its Origins, Consequences, and Molecular Mechanisms." *Frontiers in Cardiovascular Medicine* 9 (April). <https://doi.org/10.3389/fcvm.2022.845942>.
- Halstead, Kristen M., Elizabeth M. Wetzel, Josalyn L. Cho, and Anna E. Stanhewicz. 2023. "Sex Differences in Oxidative Stress–Mediated Reductions in Microvascular Endothelial Function in Young Adult e-Cigarette Users." *Hypertension* 80 (12): 2641–49. <https://doi.org/10.1161/HYPERTENSIONAHA.123.21684>.
- Heyde, Alexander, David Rohde, Cameron S. McAlpine, et al. 2021. "Increased Stem Cell Proliferation in Atherosclerosis Accelerates Clonal Hematopoiesis." *Cell* 184 (5): 1348–1361.e22. <https://doi.org/10.1016/j.cell.2021.01.049>.

- Hilgendorf, Ingo, Filip K. Swirski, and Clinton S. Robbins. 2015. "Monocyte Fate in Atherosclerosis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 35 (2): 272–79. <https://doi.org/10.1161/ATVBAHA.114.303565>.
- Jensen, M. K., P. de Nully Brown, B. V. Lund, O. J. Nielsen, and H. C. Hasselbalch. 2001. "Increased Circulating Platelet-Leukocyte Aggregates in Myeloproliferative Disorders Is Correlated to Previous Thrombosis, Platelet Activation and Platelet Count." *European Journal of Haematology* 66 (3): 143–51. <https://doi.org/10.1034/j.1600-0609.2001.00359.x>.
- Jeong, Mira, Hyun Jung Park, Hamza Celik, et al. 2018. "Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo." *Cell Reports* 23 (1): 1–10. <https://doi.org/10.1016/j.celrep.2018.03.025>.
- Kessler, Michael D., Amy Damask, Sean O’Keeffe, et al. 2022. "Common and Rare Variant Associations with Clonal Haematopoiesis Phenotypes." *Nature* 612 (7939): 301–9. <https://doi.org/10.1038/s41586-022-05448-9>.
- Kim, Ki-Wook, Stoyan Ivanov, and Jesse W. Williams. 2020. "Monocyte Recruitment, Specification, and Function in Atherosclerosis." *Cells* 10 (1): 15. <https://doi.org/10.3390/cells10010015>.
- Kumar, Premranjan, Chun Liu, Jean W. Hsu, et al. 2021. "Glycine and N-acetylcysteine (GlyNAC) Supplementation in Older Adults Improves Glutathione Deficiency, Oxidative Stress, Mitochondrial Dysfunction, Inflammation, Insulin Resistance, Endothelial Dysfunction, Genotoxicity, Muscle Strength, and Cognition: Results of a Pilot Clinical Trial." *Clinical and Translational Medicine* 11 (3): e372. <https://doi.org/10.1002/ctm2.372>.
- Levin, Michael G., Tetsushi Nakao, Seyedeh M. Zekavat, et al. 2022. "Genetics of Smoking and Risk of Clonal Hematopoiesis." *Scientific Reports* 12 (1): 7248. <https://doi.org/10.1038/s41598-022-09604-z>.
- Li, Jieliang, Luong Huynh, William D. Cornwell, et al. 2021. "Electronic Cigarettes Induce Mitochondrial DNA Damage and Trigger TLR9 (Toll-Like Receptor 9)-Mediated Atherosclerosis." *Arteriosclerosis, Thrombosis, and Vascular Biology* 41 (2): 839–53. <https://doi.org/10.1161/ATVBAHA.120.315556>.
- Liao, Min, Ruiqing Chen, Yang Yang, et al. 2022. "Aging-Elevated Inflammation Promotes DNMT3A R878H-Driven Clonal Hematopoiesis." *Acta Pharmaceutica Sinica B* 12 (2): 678–91. <https://doi.org/10.1016/j.apsb.2021.09.015>.
- Machado, Regina C.B.R., Heber O. Vargas, Marcela M. Baracat, et al. 2020. "N-Acetylcysteine as an Adjunctive Treatment for Smoking Cessation: A Randomized Clinical Trial." *Brazilian Journal of Psychiatry* 42 (5): 519–26. <https://doi.org/10.1590/1516-4446-2019-0753>.
- Mayle, Allison, Liubin Yang, Benjamin Rodriguez, et al. 2015. "Dnmt3a Loss Predisposes Murine Hematopoietic Stem Cells to Malignant Transformation." *Blood* 125 (4): 629–38. <https://doi.org/10.1182/blood-2014-08-594648>.
- Miller, Peter G., Dandi Qiao, Joselyn Rojas-Quintero, et al. 2022. "Association of Clonal Hematopoiesis with Chronic Obstructive Pulmonary Disease." *Blood* 139 (3): 357–68. <https://doi.org/10.1182/blood.2021013531>.
- Rossi, Derrick J., David Bryder, and Irving L. Weissman. 2007. "Hematopoietic Stem Cell Aging: Mechanism and Consequence." *Experimental Gerontology* 42 (5): 385–90. <https://doi.org/10.1016/j.exger.2006.11.019>.
- Schmaal, Lianne, Lotte Berk, Kai P. Hulstijn, Janna Cousijn, Reinout W. Wiers, and Wim van den Brink. 2011. "Efficacy of N-Acetylcysteine in the Treatment of Nicotine Dependence: A

- Double-Blind Placebo-Controlled Pilot Study.” *European Addiction Research* 17 (4): 211–16. <https://doi.org/10.1159/000327682>.
- Tajiri, Kazuko, Yasuhito Suehara, Toshio Suzuki, and Ikuo Sekine. 2023. “Clonal Hematopoiesis and Associated Cardiovascular Diseases.” *Japanese Journal of Clinical Oncology* 53 (3): 187–94. <https://doi.org/10.1093/jjco/hyac210>.
- Tomas, Lukas, Filip Prica, and Christian Schulz. 2021. “Trafficking of Mononuclear Phagocytes in Healthy Arteries and Atherosclerosis.” *Frontiers in Immunology* 12 (October): 718432. <https://doi.org/10.3389/fimmu.2021.718432>.
- Wang, Wei, Wenli Liu, Trevor Fidler, et al. 2018. “Macrophage Inflammation, Erythrophagocytosis, and Accelerated Atherosclerosis in Jak2V617F Mice.” *Circulation Research* 123 (11): e35–47. <https://doi.org/10.1161/CIRCRESAHA.118.313283>.
- You, Xiaona, Fabao Liu, Moritz Binder, et al. 2022. “Asxl1 Loss Cooperates with Oncogenic Nras in Mice to Reprogram the Immune Microenvironment and Drive Leukemic Transformation.” *Blood* 139 (7): 1066–79. <https://doi.org/10.1182/blood.2021012519>.
- Yu, Zhi, Trevor P. Fidler, Yunfeng Ruan, et al. 2023. “Genetic Modification of Inflammation- and Clonal Hematopoiesis–Associated Cardiovascular Risk.” *The Journal of Clinical Investigation* 133 (18). <https://doi.org/10.1172/JCI168597>.