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The impact of sterile chronic inflammation on bone marrow and extramedullary hematopoiesis

Vliv sterilního chronického zánětu na hematopoézu v kostní dřeni a extramedulární hematopoézu

Doctoral thesis

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Prague, 2025

Declaration:

I hereby declare that this thesis is a presentation of my original research, where I referenced all the relevant literary sources. I also state that neither this work, nor its substantial part, was used for the award of academic degree or diploma in the past. I would like to state that in this thesis, DeepL Write AI was used to improve English grammar.

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Ing.Maria Kuzmina

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I moved to the Czech Republic in September 2012 to pursue my university studies. Since then, it has been nearly 13 years of continuous education—a journey that has shaped both my personal and professional growth. The Czech Republic will always be a place I associate with these transformative years.

My fascination with biology and chemistry has been with me since childhood, and with many doctors in my family, I was always encouraged to explore these fields. This passion initially led me to study chemistry at the University of Chemistry and Technology in Prague, where I completed my bachelor's and master's degrees. At the time, I believed I had studied enough and envisioned a peaceful, settled life. However, it didn't take even a year for me to start missing the process of learning and gaining new knowledge. It was then, that my former mentor, Dr. Jana Balounová, inspired me to pursue a PhD.

The PhD journey has been long and challenging, yet incredibly rewarding. It has taught me invaluable lessons—far more than what could fit within the pages of this thesis. It completely reshaped my career path, shifting my focus from chemistry to immunology. I still vividly remember failing my first immunology exam, which only motivated me to take as many immunology courses as possible at Charles University to catch up with my peers. Over time, I grew from someone hesitant to make independent decisions to a confident, self-reliant scientist. Every challenge and troubleshooting experience brought me to where I am today, and I am deeply grateful to Charles University and my supervisor, Meritxell Alberich-Jordà, for believing in me and guiding me through this journey, even when it wasn't always easy. I truly appreciate the opportunities Meri provided me with, the knowledge she shared, and the way she pushed me to become an independent researcher.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
A2AR	adenosine receptor 2A
APC	antigen-presenting cell
BM	bone marrow
CAF	cancer-associated fibroblast
cAMP	cyclic adenosine monophosphate
CCL21	CC-chemokine ligand 21
CCR7	CC-chemokine receptor 7
cDC	conventional DC
CLP	common lymphoid progenitor
CMO	chronic multifocal osteomyelitis
CMO	chronic multifocal osteomyelitis
CMP	common myeloid progenitor
CRMO	chronic recurrent multifocal osteomyelitis
CXCL8	chemokine (C-X-C motif) ligand 8
DAMP	damage-associated molecular patterns
DC	dendritic cell
EC	endothelial cell
EMH	extramedullary hematopoiesis
Foxp3	forkhead box P3
GALT	gut-associated lymphoid tissue

GMP	granulocyte–macrophage progenitor
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
IFNγ	interferon gamma
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
iTreg	induced T regulatory cell
JAK	Janus kinase
KO	knockout
LAG3	lymphocyte-activation gene 3
LPS	lipopolysaccharide
MEP	megakaryocyte–erythrocyte progenitor
MFI	mean fluorescence intensity
MHCII	major histocompatibility complex class II
MLL	mixed lineage leukemia
MMP9	matrix metalloproteinase 9
mPB	mobilized peripheral blood
MPP	multipotent progenitor
MSC	mesenchymal stem cell
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NK	natural killer
NRP1	neuropilin-1

NSAID	nonsteroidal anti-inflammatory drug
nTreg	natural T regulatory cell
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PB	peripheral blood
PD-L1	programmed death-ligand 1
PRR	pattern recognition receptor
pTreg	peripherally-induced T regulatory cell
ROS	reactive oxygen species
SCF	stem cell factor
TGF-β	transforming growth factor β
TIM-3	T-cell immunoglobulin and mucin domain 3
TNF- α	tumor necrosis factor α
Tr1	type 1 regulatory T cell
Treg	T regulatory cell
WT	wild type

ABSTRACT

Antigen presentation plays a critical role in activating the adaptive immune system. Dendritic cells (DCs) are traditionally recognized as professional antigen-presenting cells (APCs), proficient in acquiring and processing antigens for presentation via major histocompatibility complex class II (MHCII). However, increasing evidence suggests that other cell types, including epithelial cells, fibroblasts, and hematopoietic stem cells (HSCs), can also function as non-professional APCs, particularly under inflammatory conditions. HSCs are crucial for the continuous production of blood cells throughout life, residing primarily in the bone marrow (BM) and maintaining haematopoiesis. Under stress conditions such as acute or chronic inflammation, cancers, or other pathological states, HSCs can migrate to extramedullary sites, such as the spleen and liver, where they perform extramedullary hematopoiesis (EMH). While much is known about BM-resident HSCs, far less is understood about the behaviour and function of HSCs found outside of the BM. In this thesis, we investigated the effects of chronic inflammation on EMH HSCs using a mouse model of chronic multifocal osteomyelitis (CMO). Our analyses revealed that EMH HSCs exhibit an upregulation of MHCII, MHCII-related genes, and immunoregulatory markers—including *CD274, Icosl, IL10r,* and *TGFbr*—suggesting an enhanced capacity for antigen presentation and potential interactions with regulatory T cells (Tregs). We hypothesize that this gene signature reflects a functional crosstalk between HSCs and Tregs. Furthermore, our research identified a unique subtype of EMH HSCs capable of inducing Tregs *in vitro* and suggesting that Tregs play a protective role in EMH *in vivo* by safeguarding HSCs from the harmful effects of chronic inflammation.

The research is framed within a broader investigation using the same CMO model and is complemented by two co-authored publications. One explores the impact of chronic inflammation on HSCs within the BM, where we found that, although the HSC compartment is expanded, these HSCs are functionally impaired via the IL-6/JAK/STAT3 axis, leading to reduced HSC functionality. The second publication investigates the influence of murine genetic background on CMO disease progression. Together, these studies provide valuable insights into the complex interactions between HSCs, immune cells, and immune regulation in the context of sterile chronic inflammation.

ABSTRAKT CZ

Prezentace antigenu hraje klíčovou roli v aktivaci adaptivního imunitního systému. Dendritické buňky (DB) jsou tradičně považovány za profesionální antigen-prezentující buňky, které jsou schopné efektivně získávat a zpracovávat antigeny pro prezentaci prostřednictvím hlavního histokompatibilního komplexu třídy II (MHCII). Ukazuje se však, že i jiné buněčné typy, včetně epiteliálních buněk, fibroblastů a hematopoetických kmenových buněk (HKB), mohou také prezentovat antigen, zejména za zánětlivých podmínek. HKB jsou klíčové pro kontinuální tvorbu krevních buněk během celého života a primárně sídlí v kostní dřeni (KD). Při stresových podmínkách, jako je akutní či chronický zánět, rakovina nebo jiné patologické stavy, mohou HKB migrovat do extramedulárních míst, jako je slezina nebo játra, kde vykonávají extramedulární hematopoézu (EMH). Zatímco fungování HKB v kostní dřeni je dobře popsáno, podstatně méně se ví o HKB nacházejících se mimo KD. V této dizertační práci jsme zkoumali vliv chronického zánětu na EMH HKB pomocí myšího modelu chronické multifokální osteomyelitidy (CMO). Naše data ukázala, že EMH HKB vykazují zvýšenou expresi MHCII, genů souvisejících s MHCII a imunoregulačních molekul, včetně CD274, Icosl, IL10r a TGFbr, což naznačuje zvýšenou schopnost prezentace antigenu a potenciální interakci HKB s regulačními T lymfocyty (Treg). Dále jsme identifikovali unikátní populaci EMH HKB, která je schopná indukovat Treg *in vitro*, a naše výsledky naznačují, že Treg hrají protektivní roli v EMH *in vivo* tím, že chrání HKB před škodlivými účinky chronického zánětu. Tento výzkum je součástí širší studie využívající stejný model CMO a je doplněn dvěma spoluautorskými publikacemi. První publikace se zaměřuje na vliv chronického zánětu na HKB v kostní dřeni, kde jsme zjistili, že ačkoliv se fenotypická populace HKB rozšiřuje, tyto HKB nejsou plně funkční, v důsledku aktivace IL-6/JAK/STAT3 dráhy. Druhá publikace zkoumá vliv genetického pozadí myší na progresi CMO. Tyto studie společně poskytují cenné poznatky o komplexních interakcích mezi HKB, imunitními buňkami a imunitní regulací v kontextu sterilního chronického zánětu.

1. INTRODUCTION

1.1. The hematopoietic system

The hematopoietic system is a vital component of the body, responsible for the continuous production of blood cells—including red blood cells, white blood cells, and platelets. This system is essential for effective oxygen transport, robust defense against pathogens, and proper hemostasis (blood clotting), all of which are critical for maintaining overall health. These cells are produced in the BM through a process known as hematopoiesis, which relies on HSCs—phenotypically defined as Lin^-, c-Kit^+, Sca-1^+, $CD48^-$, $CD150^+$—that reside at the apex of the hematopoietic hierarchy [1].

In this section, I will introduce key components of the hematopoietic system—specifically HSCs, granulocytes, DCs (including non-professional APCs), and Tregs—as they have been central to my PhD research.

1.1.1. Hematopoietic stem cells

HSCs possess unique properties essential for sustaining continuous blood cell production. First, they have the ability to self-renew, enabling them to produce identical daughter cells and replenish the stem cell pool. Second, they exist in the G_0 phase of the cell cycle, also known as quiescence—a dormant state that prevents excessive proliferation and minimizes the risk of mutation accumulation, which could otherwise lead to blood malignancies. Third, HSCs are multipotent, meaning they can differentiate into all blood cell lineages (Figure 1).

Under steady-state conditions, the majority of blood cell production is maintained by the highly proliferative HSC progeny, the progenitors [2, 3]. However, when HSCs detect environmental cues and, based on the nature, duration, and concentration of these signals that have a potential to trigger demand-adapted haematopoiesis, HSCs exit quiescence and engage in proliferation, differentiation, or self-renewal as needed [4]. To produce mature blood cells HSCs undergo multiple stages of differentiation. The first stage involves the formation of multipotent progenitors (MPPs, phenotypically defined as Lin-, c-Kit+, Sca-1+, CD48+, CD150-), which lose their ability to self-renew but retain the capacity to differentiate into multiple hematopoietic lineages. As differentiation progresses, MPPs give rise to oligopotent progenitors, which are already lineage restricted [5] [6]. For example, common myeloid

progenitors (CMPs) can differentiate into granulocyte-monocyte progenitors (GMPs), which produce granulocytes and monocytes, or megakaryocyte/erythrocyte progenitors (MEPs), which give rise to megakaryocytes and erythrocytes. Similarly, common lymphoid progenitors (CLPs) differentiate into lymphoid cells, such as B cells, T cells, and natural killer (NK) cells.

Recent findings from single-cell RNA sequencing and *in vivo* lineage tracing have challenged the traditional view of hematopoietic differentiation as a rigid, tree-like branching process, revealing instead that it operates as a continuum [7, 8]. In this model, cell types can emerge from multiple sequences of molecular events, leading to "loops" where distinct branches converge, and suggesting that lineage commitment remains adaptable even at later stages of development. Collectively, these dynamic properties enable HSCs to sustain long-term blood production, preserve their functionality, and prevent exhaustion.

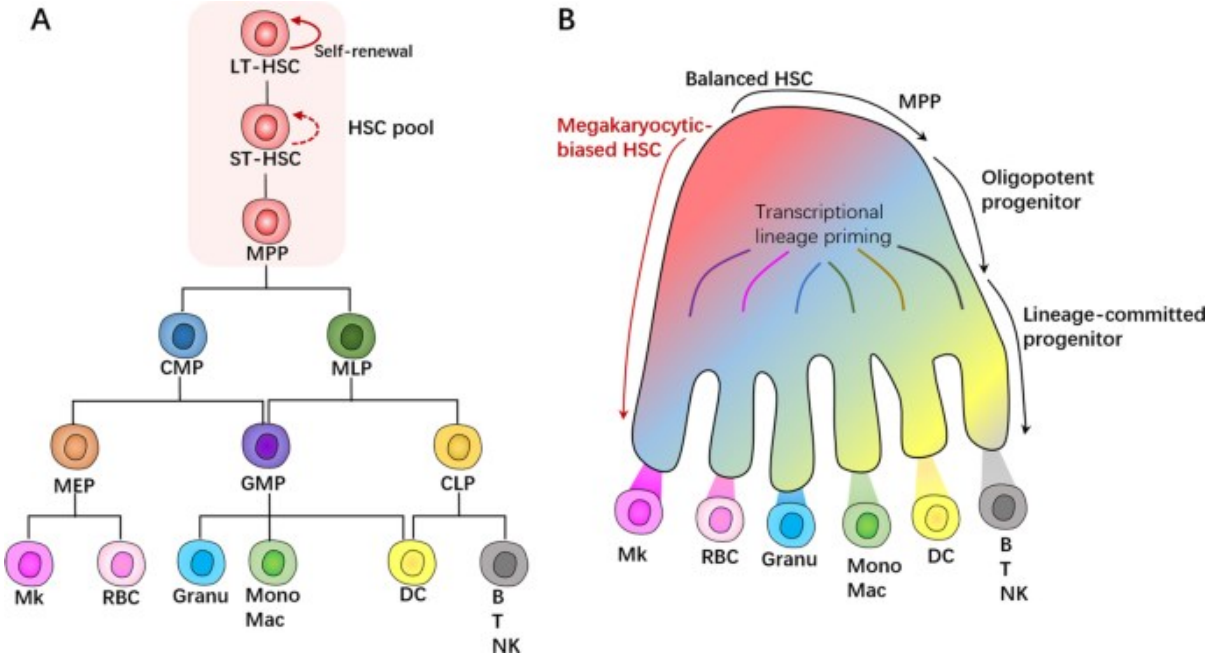


Figure 1. Hematopoietic tree*. (adopted from: Zhang, P., et al., Single-cell RNA sequencing to track novel perspectives in HSC heterogeneity. Stem Cell Res Ther, 2022. 13(1): p. 39)[9]*

(A) Classical view of hematopoietic hierarchical tree; (B) Schematic representation of HSC commitment, based on recent transcriptomic data and in vivo tracing experiments.

1.1.2. Granulocytes

Granulocytes are innate immune cells characterized by specialized secretory granules and vesicles within their cytoplasm. They originate in the BM and circulate through the bloodstream, where they play a crucial role in immunosurveillance. Based on Romanowsky

staining, granulocytes are categorized into three types: neutrophils, eosinophils and basophils [10]:

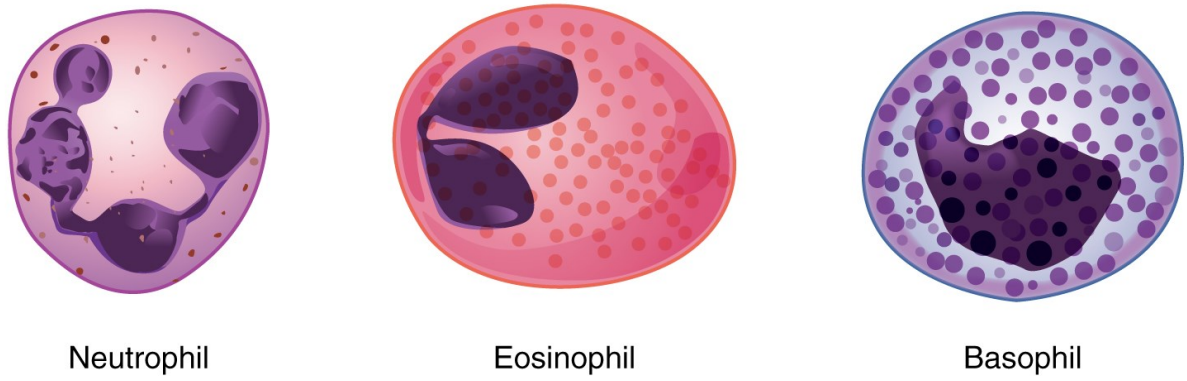


Figure 2. Granulocyte subtypes. *(adopted from: Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013)*

Basophils and eosinophils are both minor subsets of circulating granulocytes, constituting only a small fraction of peripheral blood leukocytes. Despite their low abundance, they play crucial roles in type 2 immunity by producing cytokines and inflammatory mediators. Both cell types predominantly secrete IL-4, IL-5, and IL-13, which promote eosinophil recruitment, Th2 polarization, and allergic inflammation. Eosinophils, distinguished by their bilobed nucleus and large eosinophilic granules, contribute to parasite defense and allergic reactions by releasing cytotoxic enzymes and reactive oxygen species upon activation [11]. Basophils, the rarest granulocyte subset (~0.5% of leukocytes), amplify type 2 immune responses by degranulating upon activation and releasing histamine, proteases, and lipid mediators, which enhance inflammation and allergic responses [12]. While their role in immune regulation is recognized, basophils and eosinophils remain less studied than neutrophils, and their precise contributions to immune homeostasis and disease pathogenesis continue to be explored.

Neutrophils represent both the most abundant cell type in human blood and the most extensively studied granulocyte subtype. Their development begins with HSCs, that commit to myeloid-biased progenitors (MPP3), which give rise to GMPs, followed by differentiation into myeloblasts and subsequent maturation into granulocytes [13]. As they mature, neutrophil nuclei transition from a round shape to a banded, segmented form, marking progressive stages of differentiation.

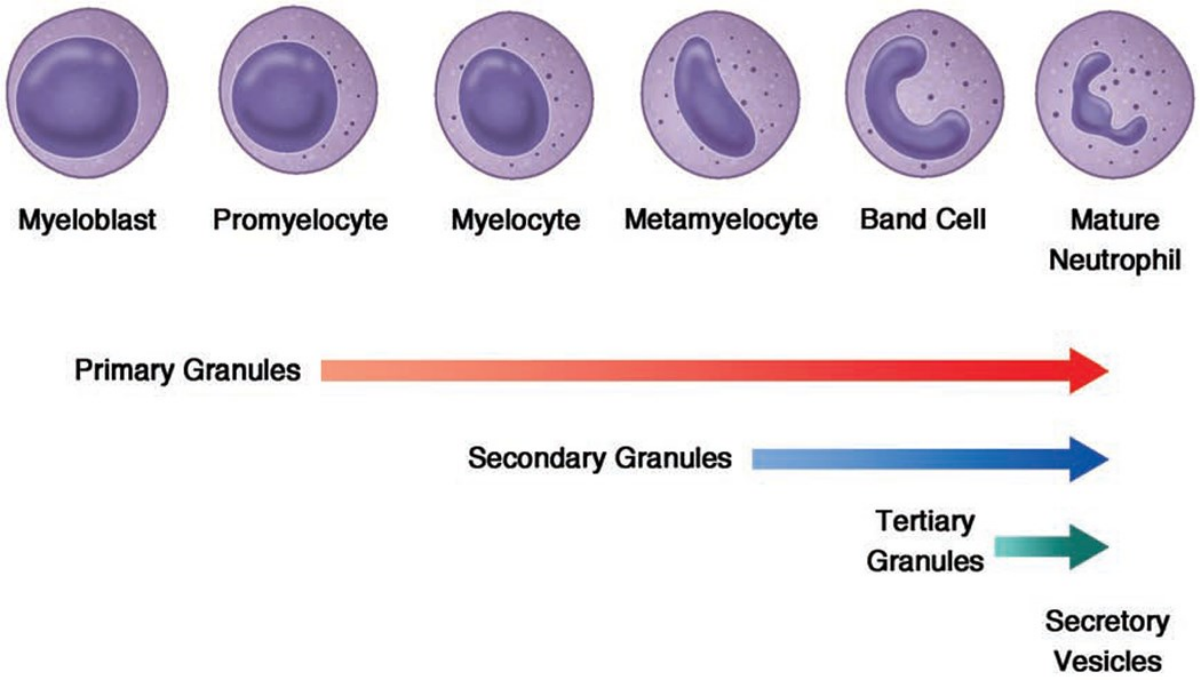


Figure 3. Neutrophil developmental stages*. (adopted from: Lehman, H.K. and B.H. Segal, The role of neutrophils in host defense and disease. J Allergy Clin Immunol, 2020. 145(6): p. 1535-1544)[14]*

Each day, the BM produces approximately 10^{11} neutrophils [15], which are short-lived (6–8 hours) and released into circulation in an inactive state [16], patrolling the body for signs of microbial infection [17]. Neutrophils possess a wide array of pattern recognition receptors (PRRs), including Toll-like, C-type lectin, and NOD-like receptors that enable them to detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [18]. Upon encountering pathogens, neutrophils mount a rapid immune response through several mechanisms:

Phagocytosis: a process by which pathogens are engulfed and subsequently degraded via an acidic environment and specialized enzymes [19];

Neutrophil degranulation: a process by which neutrophils release pro-inflammatory factors from primary, secondary, tertiary, and secretory granules. Some of these factors (e.g., defensins, myeloperoxidase, lysozyme) contribute directly to pathogen killing, while others facilitate matrix degradation (matrix metalloproteinase 9; MMP9) or support the oxidative burst (lactoferrin) [20];

Neutrophil extracellular traps (NETs): when the pathogen is too large to be ingested, neutrophils release web-like structures composed of chromatin and antimicrobial proteins that neutralize pathogens extracellularly [21];

Production of reactive oxygen species (ROS) (also known as oxidative burst): in neutrophils, the activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase converts oxygen into reactive oxygen species, which help kill invading microbes [22].

Through these coordinated responses, neutrophils serve as a critical first line of defense against a variety of pathogens.

For a long time, neutrophils were recognized solely for their pathogen-killing properties. However, it has now become evident, that dysregulated neutrophils can be key mediators of various pathological conditions, including autoimmune diseases and cancer [23]. Once activated, neutrophils contribute to inflammation by synthesizing and secreting cytokines, chemokines, leukotrienes, and prostaglandins. For instance, the chemokine CXCL8 produced by these cells recruits additional neutrophils to sites of inflammation [24], while cytokines such as IL-1, IL-6, IL-12, transforming growth factor β (TGF-β), and tumor necrosis factor α (TNF-α) activate both neutrophils and other immune cells, thus amplifying inflammatory response.

In conclusion, granulocytes are key players in the innate immune response, with neutrophils being the most extensively studied subtype due to their abundance and involvement in numerous diseases. In this thesis, we will specifically explore how different murine genetic backgrounds shape neutrophil responses and elucidate how chronic inflammation impacts HSC function as a consequence of granulocyte dysregulation.

1.1.3. Dendritic cells

Dendritic cells (DCs) are another myeloid-derived cell type in the BM and are often described as a bridge between innate and adaptive immunity. These potent antigen-presenting cells (APCs) are widely distributed throughout the body, continuously surveying for potentially harmful materials. Under steady-state conditions, murine conventional DCs (cDCs) can be broadly categorized into two groups: lymphoid DCs and migratory DCs [25]. Lymphoid DCs primarily reside in the spleen and draining lymph nodes, sampling antigens from the bloodstream and lymph for T cell presentation. By contrast, migratory DCs traffic from

peripheral tissues such as skin, lung, liver, kidney and intestinal tract to the draining lymph node already loaded with antigen [26].

Like neutrophils, DCs possess various PRRs that enable them to detect “danger signals” from both invading pathogens and injured host cells [27]. In the absence of antigenic stimulation, DCs remain in an immature state, primarily sampling the environment through receptor-dependent endocytosis and micropinocytosis. Once they internalize antigens, they undergo maturation, characterized by upregulated MHC class II and co-stimulatory molecules (CD80, CD86) expression, increased cytokine production, and the induction of CC-chemokine receptor 7 (CCR7) [28, 29]. CCR7 interacts with its ligand, CC-chemokine ligand 21 (CCL21), on lymphatic endothelial cells, guiding DCs to lymphoid tissues where they present antigens to naïve T cells.

1.1.4. Non-professional antigen-presenting cells

Non-professional—or “amateur”—APCs refer to cells that are not classified as professional APCs but still exhibit a limited capacity for exogenous antigen presentation, typically under specific conditions [30]. Both professional and amateur APCs can internalize extracellular antigens and present them on MHC class I or class II molecules. While MHC class I is expressed by all nucleated cells and typically activates CD8+ T cells specific for antigens originating from infections or malignant transformation, MHC class II is mainly expressed on professional APCs but can appear on non-professional APCs under certain stress conditions to facilitate CD4+ T cell responses [31]. The expression of MHC class II and other antigen-processing components is regulated by pro-inflammatory cytokines mainly by interferon gamma (IFNγ), explaining how non-professional APCs might enhance antigen-presenting capabilities and subsequently modulate T cell activation [32, 33].

Among non-professional APCs, fibroblasts are frequently identified, particularly under pathological conditions such as cancer or inflammation. Antigen-presenting fibroblasts were first documented in pancreatic ductal carcinoma [34]. Since fibroblasts are a major component of solid tumors, cancer-associated fibroblasts (CAFs) are of considerable interest for their role in shaping anti-tumor T cell responses [35]. In vitro assays demonstrated that CAFs present exogenous antigens on MHC class II but lack co-stimulatory molecules like CD80, suggesting their antigen presentation likely induces T cell anergy and promotes immune tolerance [34]. In

the context of autoimmune diseases such as rheumatoid arthritis, synovial fibroblasts from human patients were also shown to activate CD4+ T cells, prompting them to produce IL-2—a key cytokine associated with T cell activation [36]. Under physiological conditions, a subset of fibroblasts in human lungs [37], liver [38, 39] and colon [40, 41] also express MHC class II but not CD80 or CD86 co-stimulatory molecules. Instead, they express 4-1BBL, OX-40L, and CD70, enabling them to engage and activate memory T cells but not sufficiently prime naive T cells.

Another major stromal cell type capable of antigen presentation is the endothelial cells (ECs), which lines the interior of blood vessels and serves as a critical interface between the bloodstream and surrounding tissues. Under physiological conditions, liver sinusoidal ECs and human renal vascular ECs can effectively present exogenous antigens via MHC class II to CD4+ T cells [42-44]. However, ECs typically also lack the co-stimulatory molecules CD80 and CD86 [45]—required for naive T cell activation via CD28—and therefore primarily activate antigen-experienced T cells or induce tolerance [46]. *In vitro* stimulation with IFNγ and TNF can induce immunomodulatory changes in both human and mouse ECs, promoting antigen uptake, processing, and presentation [47, 48]. Like fibroblasts, ECs in tumors are of particular interest, as angiogenesis is essential for supplying cancer cells with nutrients and oxygen. Thus, the antigen-presenting abilities of ECs may significantly shape T cell responses in the tumor microenvironment.

Interestingly, MHC class II expression has also been observed on stem cells, although its functional significance remains largely unclear. However, several studies suggest that these cells may modulate T cell biology. For instance, MHCII expression on Lgr5+ intestinal stem cells appears to regulate stem cell proliferation, as the deletion of either MHCII or Tregs increases intestinal stem cell numbers [49]. In the hematopoietic system, HSPCs have recently gained attention as potential APCs, despite reports of their MHCII expression dating back to the 1980s [50]. Several studies have now shed light on the role of MHCII on HSCs. For instance, work by Dr. Haas's group demonstrated that HSPCs express MHCII at lower levels than professional APCs, with expression significantly increasing following LPS administration. Furthermore, only MHCII+ HSPCs—rather than MHCII- counterparts—exhibit genuine long-term repopulating capacity in both primary and secondary transplantation

assays. These findings align with research from Dr. Green's group, which shows that MHCIIhigh HSCs represent a highly quiescent subset, resistant to stress-induced proliferation—a hallmark of true stem cell behavior [51]. Of note, CD150high BM Tregs have been shown to support HSC quiescence as their depletion resulted in an increased number of HSCs [52].

Furthermore, Dr. Haas's group proposed that MHCII on HSPCs functions as a quality control mechanism, removing aberrant stem cells and reducing the likelihood of leukemic transformation while preserving BM integrity [53]. Specifically, when HSPCs present altered self-antigens via MHCII, they engage in a bidirectional interaction with CD4+ T cells. As a result, HSPCs differentiate into mature cells, effectively removing the aberrant stem cell from the pool. Simultaneously, naive T cells become type 1 regulatory T cells (Tr1), which are forkhead box P3 (Foxp3) negative rather than Foxp3 positive and express IL-10 as well as inhibitory molecules such as programmed death-ligand 1 (PD-L1), lymphocyte-activation gene 3 (LAG3), and T-cell immunoglobulin, and mucin domain 3 (TIM3). It is hypothesized that this immunosuppressive phenotype may help protect the healthy BM from collateral damage. Additional analyses of The Cancer Genome Atlas showed elevated scores for both MHCII expression and immunosuppressive markers in leukemic stem cells. To confirm these observations, the authors used mixed lineage leukemia (MLL)-AF9 mouse leukemia model co-expressing ovalbumin (MLL-AF9 OVA), transferring these transformed cells along with OT-II CD4+ T cells. In this setting, the OT-II T cells adopted an immunoregulatory program upon interacting with MLL-AF9 OVA leukemic cells, facilitating the elimination of those aberrant cells.

In contrast, the group of Dr. Wang investigated MHCII expression on aged HSCs and reported findings that diverge from those of Haas and colleagues. They observed that DNA mutations accumulating with age elevate MHCII levels in HSCs. These MHCIIhigh aged HSCs are subsequently recognized by BM Tregs, leading to HSC clonal expansion. As a result of the HSC-Treg interaction HSCs receive anti-apoptotic priming mediated by gap junction transfer of cyclic adenosine monophosphate (cAMP). According to the authors, this aged HSC–BM Treg interaction provides pro-survival signals, giving a clonal advantage of MHCIIhigh HSCs over their MHCII- counterparts [54].

It has now become evident that multiple cell types can shape T cell responses by acquiring antigen-presenting capabilities, particularly under pathophysiological conditions such as inflammation or cancer. Furthermore, the anatomical context—for example, in the intestine or lungs—can influence the ability of certain cells to present antigens. Given these insights, additional research on MHCII expression in non-professional APCs is needed to determine with which T cells they engage, what co-stimulatory molecules they utilize, and how they process antigen. In this thesis, we will investigate how HSPCs modulate T cell responses through MHCII interactions and how these interactions, in turn, affect HSPCs at peripheral sites under chronic inflammatory conditions.

1.1.5. T regulatory cells

Tregs are a critical subset of T lymphocytes characterized by the expression of the Foxp3 transcription factor [55, 56] and a high-affinity IL-2 receptor alpha chain (CD25) [57]. They play a central role in mediating peripheral tolerance, a mechanism through which the immune system prevents excessive or autoreactive responses to both foreign and self-antigens.

Two main types of Tregs have been identified to date:

- **Natural Tregs** (nTregs): developed in the thymus during positive and negative selection. They typically exhibit relatively high-affinity TCRs for self-antigens and are essential for preventing autoimmunity;
- **Induced Tregs** (iTregs): also termed peripheral Tregs (pTregs) arise from naive CD4+ T cells in the periphery in response to suboptimal TCR signaling [58, 59] and exogenous antigens in an environment rich in TGF-β and IL-2, or *in vitro* by stimulating CD4+ T cells with these two cytokines [60, 61]. iTregs are thought to be especially important at mucosal sites, such as in the gut, lungs, and skin and during inflammation.

Among the iTregs, two main subsets have been described based on their induction cytokines:

- **Type 1 regulatory T cells** (Tr1): induced by IL-10 and not expressing Foxp3 [62, 63];
- **T helper 3** (Th3): induced by TGF-β and expressing Foxp3 [64].

Although IL-10 and TGF-β are major drivers of iTreg development, IL-4 and IL-13 can also induce Foxp3+ Tregs from Foxp3- naive T cells in a process independent of TGF-β and IL-10.

Both IL-4 and IL-13 signal through the IL-4 receptor alpha chain, emphasizing the significance of this receptor in Treg development within the periphery [65]. Interestingly, co-stimulation through CD28 is essential for the generation of nTregs, whereas iTregs have been shown to develop in the periphery independently of CD28 co-stimulation [59, 66]. Moreover, iTregs generated *in vitro* without CD28 co-stimulation have been shown to remain functionally competent *in vivo* after adoptive transfer [67].

In mice, nTregs and iTregs can be distinguished by the expression of the transcription factor Helios (iTregs are negative for Helios) [68] and the immunoregulatory receptor neuropilin-1 (NRP1; iTregs are negative for NRP1) [69]. However, in humans, no definitive markers currently exist to discriminate between these two Treg subsets.

Several cell types have been identified as capable of inducing *de novo* iTreg differentiation. Monocyte-derived DCs, including plasmacytoid DCs, have been shown to facilitate iTreg formation [70-72]. Notably, DCs within the gut-associated lymphoid tissue (GALT) exhibit exceptional efficiency in inducing iTregs [73-75] as do DCs residing in the tumor microenvironment [76]. Interestingly, Tregs themselves can induce DCs to adopt a tolerogenic phenotype, creating a positive feedback loop in GALT that, in the presence of TGF-β, further amplifies iTreg induction [77]. Additionally, a subset of macrophages within the lamina propria has been demonstrated to promote *de novo* Foxp3+ Treg differentiation through a mechanism dependent on retinoic acid, TGF-β, and IL-10 [78].

Tregs employ multiple mechanisms to ensure peripheral tolerance, each contributing to immune suppression in distinct ways:

1) **Inhibitory cytokine secretion** – Tregs produce and secrete key immunosuppressive cytokines, including IL-10, TGF-β, and IL-35, which are considered primary mediators of Treg-induced suppression. These cytokines modulate the activity of conventional T cells, DCs, and other immune cells, thereby maintaining immune homeostasis [79-81];
2) **Cytolysis** – Tregs can mediate direct cytotoxicity through the production of granzymes. In humans, this is primarily facilitated by Granzyme A, while in mice,

Granzyme B plays a predominant role. These granzymes enable Tregs to induce apoptosis in target cells in a granzyme-dependent manner [82, 83];

3) **Metabolic disruption** – Tregs can induce metabolic disruption through several mechanisms [79-81]:
    - **IL-2 deprivation** – Tregs constitutively express high levels of CD25, allowing them to sequester IL-2 from the microenvironment, thereby depriving conventional T cells of this crucial survival signal and leading to apoptosis.
    - **Adenosine-mediated immunosuppression** – Tregs express ectonucleotidases CD39 and CD73, which catalyze the conversion of extracellular ATP into adenosine. Adenosine then binds to the adenosine receptor 2A (A2AR) on conventional T cells, suppressing their activation.
    - **cAMP transfer** – Tregs can transfer the inhibitory second messenger cAMP into conventional T cells via gap junctions, further dampening their activation and proliferation.
4) **Modulation of APC function** – Tregs directly suppress APCs by engaging inhibitory receptors. CTLA-4 on Tregs binds to CD80/CD86 on APCs, leading to downregulation of co-stimulatory signals necessary for T cell activation. Additionally, Tregs express LAG-3, which interacts with MHCII on APCs, further inhibiting their ability to prime conventional T cells [79-81].

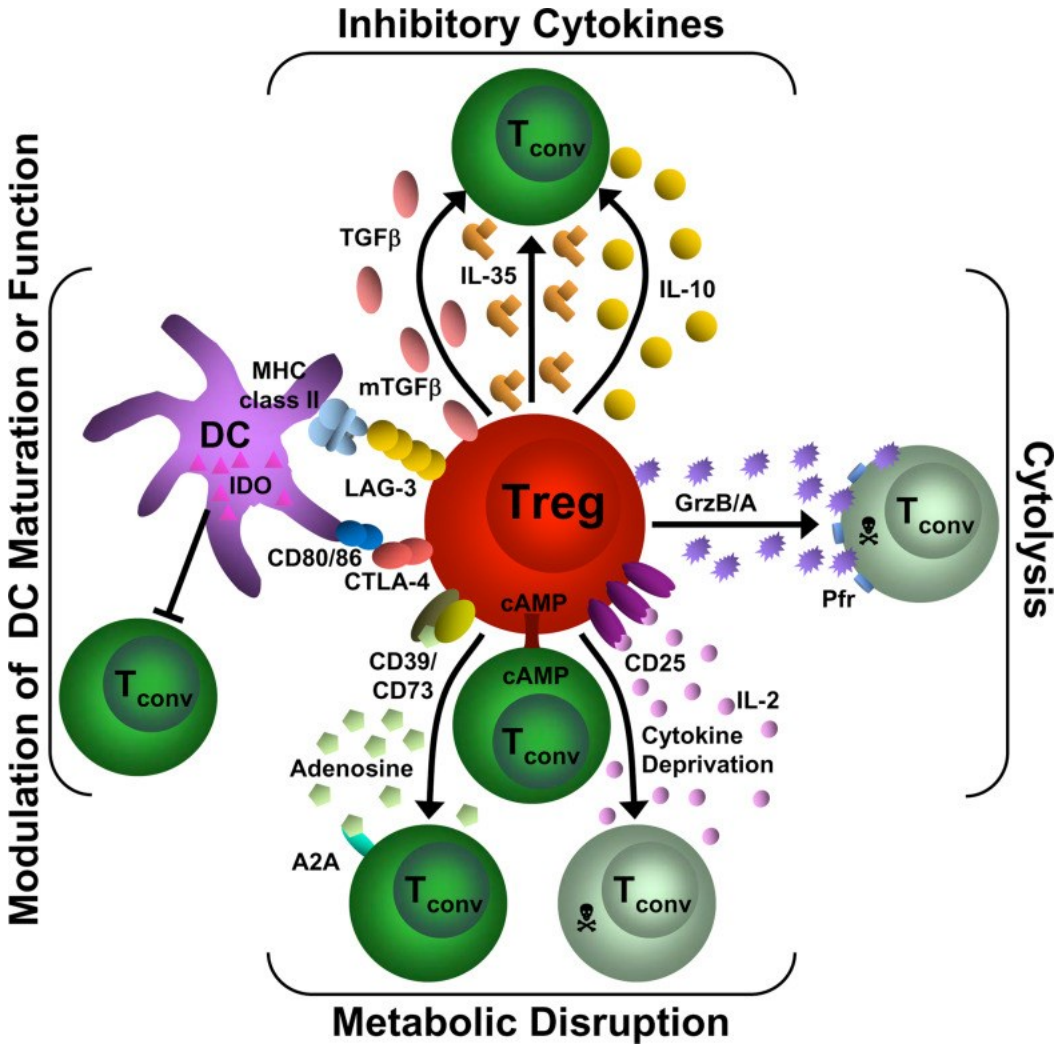


Figure 4. Tregs immunosuppressive mechanisms. *(adopted from: Workman, C.J., et al., The development and function of regulatory T cells. Cell Mol Life Sci, 2009. 66(16): p. 2603-22)[84]*

Altogether, these findings provide substantial evidence of the critical role Tregs play in maintaining peripheral tolerance. They highlight the diverse mechanisms by which Tregs suppress immune responses and emphasize the existence of two distinct types of Tregs: nTregs and iTregs. While both subsets contribute to immune regulation, they differ in their origin, development, and functional specialization. nTregs primarily develop in the thymus and are essential for controlling autoimmunity by recognizing self-antigens, whereas iTregs arise in the periphery in response to environmental or inflammatory cues and play a crucial role in regulating immune responses at mucosal surfaces and in inflamed tissues.

1.2. Inflammation

Inflammation represents a defence mechanism employed by the immune system to eliminate harmful pathogens and foreign substances. It can be classified into two forms: acute and chronic, each with distinct triggers and outcomes. Acute inflammation begins rapidly, escalates in severity over a short period, and typically resolves within a few days [85, 86]. It is often initiated during infections through the interaction between PRRs on immune cells and conserved PAMPs found on pathogens. Acute inflammation can also be triggered by DAMPs released in response to physical, chemical, or metabolic stress during cellular injury [87].

In contrast, chronic inflammation develops gradually, usually triggered by DAMPs and persists over an extended period [88, 89]. It is frequently associated with aging, as older individuals often exhibit elevated levels of circulating inflammatory cytokines, chemokines, and acute-phase proteins [88, 90, 91]. When associated with aging, chronic inflammation is referred as inflamm-aging [92]. Unlike acute inflammation, which resolves once the threat is neutralized, chronic inflammation reflects persistent immune activation, leading to collateral damage to tissues and organs over time and contributing to the progression of age-related and other pathological conditions [85, 90, 91, 93].

The main differences between acute and chronic inflammation are represented in the Figure 5.

	Acute inflammation	**Systemic chronic inflammation**
Trigger	PAMPs (infection), DAMPs (cellular stress, trauma)	DAMPs ('exposome', metabolic dysfunction, tissue damage)
Duration	Short-term	Persistent, non-resolving
Magnitude	High-grade	Low-grade
Outcome(s)	Healing, trigger removal, tissue repair	Collateral damage
Age-related	No	Yes
Biomarkers	IL-6, TNF-α, IL-1β, CRP	Silent—no canonical standard biomarkers

Figure 5. Acute inflammation versus systemic chronic inflammation. *(adopted from: Furman, D., et al., Chronic inflammation in the etiology of disease across the life span. Nat Med, 2019. 25(12): p. 1822-1832)[94]*

Since my Ph.D. thesis evolves around the topic of chronic inflammation, I will introduce this particular type of inflammation in more detail below.

1.2.1. Chronic inflammation

As it has been already mentioned above, chronic inflammation is a persistent, low-grade immune activation that plays a pivotal role in the pathogenesis of numerous age-related and lifestyle-associated diseases. It is now recognized as a major contributor to global mortality, with over 50% of all deaths attributable to inflammation-related conditions such as ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, non-alcoholic fatty liver disease, autoimmune disorders, and neurodegenerative diseases [95].

The etiology of chronic inflammation is multifactorial, involving both endogenous and exogenous contributors. A primary endogenous driver is aging [88], which induces cellular senescence—a state of cell cycle arrest accompanied by the development of a senescence-associated secretory phenotype (SASP) [96]. SASP, resulting from factors such as DNA damage, telomere dysfunction, epigenomic disruption, and oxidative stress [97], leads to the secretion of high levels of pro-inflammatory cytokines and chemokines by senescence cells, thereby establishing a systemic inflammatory milieu.

Exogenous factors further contribute to the development of chronic inflammation. Chronic infections, for example, those caused by cytomegalovirus, Epstein–Barr virus, hepatitis C virus, and HIV, have been linked to reduced lifespan and accelerated immunosenescence [98-100]. However, rather than being driven solely by chronic infections, persistent inflammation appears to result from a synergistic interplay among chronic infections, aging, genetics and lifestyle factors [101-104]. Notably, studies of non-industrialized populations reveal that despite high microbial exposure, these groups exhibit lower inflammatory markers and a reduced incidence of chronic inflammatory diseases [105-109].

Lifestyle factors are critical modulators of chronic inflammation. Populations in non-industrialized settings generally consume diets low in processed foods [110], maintain high levels of physical activity [106], and experience lower environmental pollution. In contrast, urban lifestyles—characterized by sedentary behavior, poor dietary habits, and higher exposure to pollutants—are associated with obesity, particularly increase in visceral adipose tissue [111, 112], which in turn provokes inflammatory responses. Furthermore, the accumulation of visceral adipose tissue—a defining feature of obesity—has been shown to

accelerate aging and elevate the risk of cardiometabolic, neurodegenerative, and autoimmune diseases, as well as various types of cancer [91, 111, 113]. Consequently, obesity has been linked to alterations in gut microbiota that activate pattern recognition receptors (e.g., Toll-like receptors) on immune cells [114-116]. Moreover, diets low in fruits, vegetables, and fiber but high in refined grains, alcohol [117], and ultra-processed foods [118] further contribute to the development of chronic inflammation [119, 120].

The clinical consequences of this sustained inflammatory state are profound. Chronic inflammation is implicated in the development of metabolic syndrome—a combination of hypertension, hyperglycemia, and dyslipidemia [121]—as well as type 2 diabetes, non-alcoholic fatty liver disease [122], cardiovascular disease [123], chronic kidney disease [91], various cancers [124], depression [125], neurodegenerative and autoimmune disorders [93, 126, 127], osteoporosis [128] and sarcopenia [91]. For instance, patients with autoimmune conditions like rheumatoid arthritis often exhibit insulin resistance, dyslipidemia, and hypertension, along with elevated rates of metabolic syndrome and cardiovascular complications [126, 129-131].

In summary, chronic inflammation might result from a complex interplay of aging, genetics, chronic infections, and lifestyle factors, culminating in a persistent, low-grade inflammatory state that shortens lifespan, impairs immune function, and reduces the efficacy of vaccinations. Given its profound impact on overall health, our research—as well as that of many colleagues—aims to elucidate the effects of chronic inflammation on HSCs. This topic will serve as a primary focus of this thesis.

1.2.2. CMO mouse model

To study the effects of chronic inflammation on HSCs, we utilize a mouse model of autoinflammatory condition known as Chronic Multifocal Osteomyelitis (CMO). This model, characterized by a point mutation in the *Pstpip2* gene, exhibits an expansion of the myeloid compartment and elevated levels of pro-inflammatory cytokines and chemokines [132-139], suggesting that PSTPIP2 plays a crucial role in controlling the onset of autoinflammatory disease. Unlike many of the chronic inflammatory mouse models, CMO mice develop disease spontaneously with age, with initial symptoms becoming noticeable around eight weeks [140].

The CMO phenotype is marked by osteoclast-mediated bone degradation in the hind paws and tail [139, 141]. Studies investigating the role of PSTPIP2 in CMO pathogenesis have demonstrated that the absence or mutation in the *Pstpip2* gene can initiate or exacerbate osteomyelitis. Although the precise function of PSTPIP2 requires further investigation, some evidence suggests that it regulates IL-1β and ROS production, modulates megakaryocyte and neutrophil function, and interacts with protein tyrosine phosphatases to collectively suppress inflammation [136-138, 142, 143].

The CMO mouse model has a human equivalent known as Chronic Recurrent Multifocal Osteomyelitis (CRMO), sometimes referred to as chronic non-bacterial osteomyelitis, which was first documented in 1972 [144]. CRMO typically affects young individuals and is characterized by dysregulated innate immune responses in which the innate immune system targets bone tissue, leading to inflammation in the absence of an infection [145, 146]. It is frequently accompanied by other inflammatory disorders including psoriasis and inflammatory bowel disease [147-150]. Its clinical manifestations include pain, swelling, local skin changes, and the involvement of multiple bones [151]. Due to its rarity, definitive diagnostic criteria for CRMO have not been established, and diagnosis is largely based on the exclusion of other conditions [152-154]. Moreover, there is no standardized treatment regimen for CRMO; therapy is usually tailored to the individual based on disease severity and response. Treatment options include nonsteroidal anti-inflammatory drugs (NSAIDs); if NSAIDs prove insufficient, clinicians may employ corticosteroids, bisphosphonates, TNF-α inhibitors, IL-1β inhibitors, and Janus kinase (JAK) inhibitors [151, 155-157].

To conclude, CMO represents a suitable mouse model to investigate the effects of chronic inflammation on the hematopoietic system, particularly on HSCs. In this model, *Pstpip2* expression is detectable only in mature myeloid cells [133]. This restriction allows us to exclude the possibility that the mutation intrinsically alters HSC function, thereby enabling us to focus specifically on the impact of chronic inflammation on HSC phenotype. Furthermore, clinical observations in humans indicate that the etiology and optimal treatment of CRMO remain poorly understood. Given that HSCs are the progenitors of the entire blood cell population—including neutrophils, which are believed to be the primary drivers of the disease—this model enables us to identify potential dysregulations in HSCs that may serve as

therapeutic targets to prevent myeloid expansion and, consequently, mitigate disease progression. In addition, severe cases of CRMO and the CMO mouse model often develop EMH, though proper evaluation of EMH under chronic inflammation and the role of EMH HSCs remain elusive. The current understanding of the effects of chronic inflammation on HSCs will be discussed in the subsequent sections.

1.2.3. The impact of chronic inflammation on HSCs

Inflammatory diseases arise from dysregulated immune responses that culminate in a persistent, low-grade inflammatory state. Many such conditions are of "sterile" origin, occurring in the absence of external pathogens as the immune system reacts to self-antigens. Although BM failure is rare under these circumstances, sterile inflammation is associated with cytopenias, anemia, myeloid overproduction, immunosenescence, and other hematological comorbidities. These alterations have been documented in disorders such as rheumatoid arthritis, juvenile idiopathic arthritis, systemic lupus erythematosus, colitis, and autoinflammatory conditions like gout, where IL-1 is a key pathogenic factor [158-162]. Numerous mouse models have been developed to study the impact of sterile inflammation on the hematopoietic system. For example, in an IL-23–driven **colitis model**, chronic intestinal inflammation leads to a significant expansion of the HSC pool in an IFN-γ–dependent manner, accompanied by a GM-CSF–driven bias toward myeloid differentiation and the development of EMH [163]. Similarly, in a mouse model of **Chronic Granulomatous Disease (CGD)**—a primary immunodeficiency caused by NOX gene mutations that impair microbicidal activity—there is increase in HSC proliferation, downstream progenitor expansion, and the reduction in long-term engraftment capacity, correlating with elevated IL-1β levels. Treatment with Anakinra, an IL-1β antagonist, reduced HSPC numbers in CGD mice, although the repopulating capacity of HSCs was only partially restored [164].

In **rheumatoid arthritis** models—induced either by collagen injections or through genetic manipulation—a robust overproduction of myeloid cells is also observed. This myeloid skewing is driven by intrinsic gene programs in HSCs and elevated systemic levels of cytokines such as TNF, IFN-γ, and G-CSF [165, 166]. Likewise, in a mouse model of **spondylarthritis**, HSCs are primed toward myeloid differentiation in an IL-33–GM-CSF–dependent manner, leading to the development of EMH and accumulation of myeloid progenitors at extramedullary sites, which exacerbates disease severity [167]. The **CMO** mouse model of chronic autoinflammatory bone disease similarly demonstrates expansion of HSPCs and the myeloid compartment [133, 134, 136-138];

In **systemic lupus erythematosus** mouse models, studies have observed an expansion and mobilization of HSCs from the BM, accompanied by a bias toward myeloid differentiation [168]. Unpublished data from E. Pietras's laboratory suggest that in a pristane-induced systemic lupus erythematosus model, HSC repopulating capacity is only mildly reduced despite ongoing IFN signaling [4]. Moreover, in a model of collagen-induced arthritis, HSCs activate a cell cycle arrest program associated with quiescence, even in the presence of persistent inflammation [165].

In summary, chronic inflammation—particularly in its sterile form—alters HSC lineage commitment and functionality, promotes the development of EMH, and induces myeloid skewing. Interestingly, compared to chronic infections, which impose high hematopoietic demands through rapid immune cell turnover and may directly affect HSCs, sterile inflammation appears to have a relatively milder impact on BM HSCs. This disparity may be due to differences in cytokine levels, the direct effects of pathogens on HSCs during infections, and increased hematopoietic demands associated with rapid immune cell turnover [4]. Nevertheless, further research is essential to elucidate the biological and mechanistic adaptations of HSCs under chronic inflammatory conditions, which could ultimately inform the development of targeted anti-inflammatory therapies to mitigate detrimental effects of chronic inflammation. The work presented in this thesis contributes to a better understanding of this point.

1.2.4. Extramedullary hematopoiesis

EMH refers to the production of blood cells outside of the primary niches in the BM. It can occur physiologically during embryonic development and pathologically in response to disease.

EMH in adult humans or mice is generally associated with pathological stress conditions, such as leukemia and acute or chronic inflammation [169]. Under these circumstances, BM hematopoiesis can become compromised or insufficient, prompting peripheral organs—often those involved in embryonic hematopoiesis—to compensate and support blood cell production [170]. The insufficiency of BM hematopoiesis can arise from various mechanisms that are not fully understood yet. Proposed explanations include space limitations within the BM that restrict the production of large numbers of immune cells, as well as reduced HSC 'fitness' arising from excessive proliferation induced by inflammation [171, 172].

In humans EMH is commonly observed in diseases such as myelofibrosis, diffuse osseous metastatic disease, leukemia, sickle cell disease, and thalassemia [173]. Table 1 summarizes documented cases of EMH in adults under various pathological conditions.

Main Organ or Tissue Affected	**Associated Disease(s)**	**Reference**
Brain hematoma	Congenital anomaly and anemia	[174]
Brain mass	Thalassemia	[175]
Brain	Myelofibrosis	[176]
Spinal cord	Congenital anomaly	[177]
Spinal cord	Breast cancer	[178]
Spinal cord	Thalassemia	[179]
Thorax	Thalassemia	[180, 181]
Lungs	Myelodysplasia and cirrhosis	[182]
Lungs	Myeloproliferative disease and BM fibrosis	[183]
Lungs	Sickle cell trait/β thalassemia	[184]

Lungs	Myelofibrosis with myeloid metaplasia	[185]
Liver	Myeloid metaplasia	[186]
Liver	Myelofibrosis	[187]
Liver	Myeloid metaplasia	[188]
Liver	Lupus	[189]
Spleen	Metastatic carcinoma	[190]
Spleen	Multiple myeloma	[191]
Spleen	Gray platelet syndrome with myelofibrosis	[192]
Spleen and liver	Fibrous dysplasia	[193]
Spleen and liver	Myelodysplastic syndromes	[194]

Table 1: Adult EMH in humans *(adopted from: Rivera-Torruco, G., M.O. Muench, and R. Valle-Rios, Exploring extramedullary hematopoiesis: unraveling the hematopoietic microenvironments. Front Hematol, 2024. 3)[195]*

These pathological conditions vary in nature, encompassing both non-cancerous and cancerous diseases. Among the non-cancerous disorders are lupus, Gray platelet syndrome, and fibrous dysplasia. Fully cancerous conditions include breast cancer, renal cancer, multiple myeloma, myeloproliferative disorders, myelodysplastic syndromes, and metastatic carcinoma.

In addition to human disorders, EMH has also been documented in mice under various pathological conditions. Notably, a mouse model of inflammatory arthritis has shown signs of EMH in inflamed joints. Further, in mice injected with drugs such as lipopolysaccharide (LPS) [196], cyclophosphamide [197], and 5-Fluorouracil (5-FU) [198] EMH developed in the spleen. Beyond these common treatments, murine EMH can be experimentally induced through various methods, including the administration of specific cytokines (e.g., G-CSF, IL-5, IL-6), exposure to certain drugs, or the presence of genetic mutations. Table 2 provides a comprehensive list of currently documented EMH in mice.

Drug, cytokine, or growth factor-induced EMH			
Cause	**Inducer**	**EMH Site**	**Reference**
Cytotoxicity	Cyclophosphamide	Spleen	[199]; [197]
BM failure	5-Fluorouracil	Spleen	[198]
BM failure	Nicotine	Spleen	[200]
BM failure	BMS-182248	Spleen	[201]
Myelostimulation	G-CSF	Epidermal adipose tissue	[202]
Myelostimulation	IL-5	Spleen	[203]
Myelostimulation	IL-6+sIL-6R	Spleen and liver	[204]
Myelostimulation	IL-27	Spleen	[205]
Myelostimulation	Recombinant human IL-3	Subcutaneous tissue of injection site	[206]
Myelostimulation	FGF-2	Spleen and liver	[207]
Myelostimulation	FLT3 ligand	Spleen and liver	[208]
Myelostimulation	PDGF-BB	Spleen and liver	[209]
Myelostimulation	VEGF	Spleen	[210]
Genetically-induced EMH			
Point mutation	NF-κB (*NIK*^{G855R})	Spleen	[211]
Point mutation	Jak2 ^{V617F}	Spleen	[212]
Point mutation	IDH1^{R132H}	Spleen	[213]
Deletion	Nf1	Spleen	[214]
Genetic defect-induced EMH	TET2	Liver	[215]
Deletion	PcG	Spleen and liver	[216]
Deletion	Foxp3	Spleen	[217]
Deletion	IEX-1	Spleen	[218]
Deletion	HIF-1α	Spleen	[219]

Deletion	GATA1 (HS1)	Spleen	[215]
Fusion	TEL-Syk	Spleen	[220]
Overexpression	HMGA2	Spleen	[221]
Infection or Inflammation-induced EMH			
Parasite	Plasmodium chabaudi	Spleen	[222]
Parasite	*Trypanosoma vivax*	Spleen and liver	[223]
Bacteria	*Helicobacter hepaticus*	Spleen and colon	[163]
Bacteria	*Mycobacterium bovis strain*	Spleen and peripheral blood	[224]
Bacteria	*Anaplasma phagocytophilum*	Spleen	[225]
Bacteria	*Escherichia muris*	Spleen	[226]
Bacteria	*Salmonella*	Spleen	[227]
Bacteria	*Staphylococcus aureus*	Wound	[228]
Virus	*Murine cytomegalovirus*	Spleen	[229]

Table 2: EMH in mice *(adopted from: Chiu, S.C., et al., Extramedullary hematopoiesis (EMH) in laboratory animals: offering an insight into stem cell research. Cell Transplant, 2015. 24(3): p. 349-66) [230]*

While it is well established that EMH can arise under the aforementioned pathological conditions, the precise mechanisms by which HSPCs function in peripheral organs remain poorly understood. Nonetheless, studies suggest that in the spleen, EMH predominantly localizes within the red pulp, where both mesenchymal stem cells (MSCs) and ECs produce stem cell factor (SCF), while only MSCs secrete CXCL12 to support HSPC function [231, 232]. In the liver, EMH localizes near sinusoidal endothelial cells, which produce CXCL12 creating a supportive microenvironment for HSPC proliferation and differentiation in mice [233, 234].

Moreover, immune cells also contribute to the regulation of splenic hematopoiesis. For example, NK cells appear to negatively regulate myeloid output, as a decline in NK cell

numbers in the spleen has been linked to increased myeloid progenitor populations [235]. On the other hand, some studies suggest that T cells in the spleen may act as a hematopoietic niche, with Tregs playing a role in suppressing hematopoietic cytokine–producing T cells [217, 236]. Finally, macrophages are well known to support both erythropoiesis and hematopoiesis in the spleen [196, 237, 238]

1.2.5. Extramedullary HSCs

Compared to their BM counterparts, extramedullary HSCs remain relatively understudied. Nonetheless, splenic HSCs are known to possess self-renewal and repopulating capacities comparable to those of BM HSCs [239]. Recent findings in mice indicate that, in terms of cell cycle status, splenic HSCs maintain a "pre-activated" or "alert" state in the G1 phase rather than the quiescent G_0 phase. Despite this partial activation, they do not enter the S/G2/M phases of the cell cycle without an external stimulus. This "pre-activated" state of EMH HSCs indicates their rapid-response advantage during emergency or stress conditions, enabling the swift production of cells on demand [240-242].

One gene implicated in maintaining G1 arrest is *Mtg16* as mice deficient in *Mtg16* exhibit increased numbers of splenic HSCs in the S/G2/M phases, while the cell cycle profile of BM HSCs remains unchanged. *Mtg16* encodes a transcriptional corepressor involved in cell fate decisions via the Notch signalling pathway, which has been linked to lymphoid lineage bias [243-245]. Interestingly, induced HSC mobilization or adoptive transfer of BM HSCs revealed that, once relocated to the spleen, these HSCs similarly adopt a pre-activated phenotype, suggesting that the splenic microenvironment contributes to this state [246].

A recent study by Elisa Laurenti's group [247] examined transcriptional and functional differences in human HSPCs across various extramedullary sites, including peripheral blood (PB), mobilized peripheral blood (mPB), and spleen. Their analysis revealed that under steady-state conditions, HSCs/MPPs in PB are predominantly quiescent and non-proliferative, with a strong skew toward erythroid and megakaryocytic differentiation. Notably, these cells exhibit lower repopulation ability compared to mPB or BM HSCs.

In contrast, mPB typically contains a higher proportion of HSCs and early progenitors, many of which are actively cycling, and display a lineage bias similar to BM HSCs, resulting in more balanced reconstitution. Splenic HSCs on the other hand, under steady-state conditions, tend

to remain quiescent with minimal *in situ* proliferation similarly to those in mice. Nevertheless, they demonstrate a heightened lineage priming toward erythroid and megakaryocytic differentiation, particularly under stress. Additionally, splenic HSCs exhibit distinct surface protein expression profiles indicative of altered migration and adhesion properties. Under conditions such as hereditary spherocytosis, these splenic HSCs become more active and show enhanced erythroid priming.

In summary, there is still a considerable amount of uncertainty regarding the exact role of EMH. Fundamental questions persist about the underlying mechanisms leading to EMH, the function of HSPCs in peripheral organs, how these HSPCs are maintained, whether there are differences among HSPCs at various EMH sites and whether EMH arises from residual HSPCs in the periphery from embryonic development or through their continual replenishment from the BM. These and other questions will require further investigation to be fully understood. My work presented in this thesis, brings light to some of these aspects and providing a deeper understanding of EMH HSC biology.

2. AIMS

The general goal of this thesis is to investigate how chronic inflammation influences HSCs. To explore this, we employed a mouse model of sterile chronic inflammation—known as CMO (see the "CMO mouse model" section)—which allows to study the direct effects of inflammatory signals on HSCs without the confounding influence of pathogens. Although several studies have examined the impact of chronic inflammation on BM HSCs and proposed various mechanisms, several questions remain—particularly regarding the role of additional pro-inflammatory cytokines elevated during sterile chronic inflammation. Moreover, previous reports have indicated that sterile inflammation induces EMH in both mice and, in some cases, humans. However, the functional characteristics and lineage biases of these EMH HSCs remain poorly understood. Therefore, it is essential to determine whether EMH HSCs contribute to the inflammatory process or, conversely, attempt to suppress it—a distinction that could offer additional insights into potential therapeutic options for patients with sterile chronic inflammation. Finally, the genetic background is known to significantly influence immune responses in mouse models, as demonstrated by studies of adaptive immunity. However, it remains largely unknown whether innate immune responses also vary across different genetic backgrounds. To elucidate this, we investigated whether distinct murine strains—such as BALB/c, C57BL/6N, and C57BL/6J—affect the development of autoinflammation in the CMO model, where disease progression is solely driven by a dysregulated innate immune system.

To frame our research more concretely, we established three key aims that address these scientific gaps within the context of sterile chronic inflammation.

2.1. To identify new factors contributing to the detrimental effect of chronic inflammation on BM HSCs

Although it is well established that pro-inflammatory cytokines—particularly IL-1β and IFNγ—mediate detrimental effects on HSCs during sterile chronic inflammation, the role of the IL-6/JAK/STAT axis under these conditions and its impact on BM HSCs remain largely unexplored. In our research, we specifically focused on elucidating the influence of IL-6/JAK/STAT signaling on BM HSCs in the context of sterile chronic inflammation in CMO mice.

2.2. To define the role of EMH HSCs under inflammatory conditions

CMO mice are known to develop splenomegaly with active EMH, yet the functional role of these HSCs in the spleen remains largely unexplored. Additionally, we sought to determine whether CMO mice exhibit novel sites of EMH, particularly at the primary sites of inflammation, such as in the paws and tail. This objective formed the central focus of my PhD research, which aimed to delineate the biological role of EMH HSCs under conditions of sterile chronic inflammation.

2.3. To evaluate the effect of different murine genetic backgrounds on the development of chronic inflammation in the CMO mouse model.

To assess how the murine genetic background affects the development of sterile chronic inflammation in CMO mice, we generated the CMO model on three widely used genetic backgrounds: C57Bl/6J, C57Bl/6N, and BALB/c. Although these strains are common in basic research, contradictory results are sometimes reported in similar studies using distinct murine strains. The aim of this project was to determine the extent to which the genetic background influences the chronic inflammation phenotype, and therefore we compared these strains based on typical inflammatory markers and clinical signs.

3. RESULTS AND DISCUSSION

3.1. Chronic inflammation decreases BM HSC fitness by activating the druggable Jak/Stat3 signaling pathway.

The results of this aim were published in EMBO reports. I am a co-author in this manuscript and contributed to the experimental design, performance and analysis presented in Figures 11-13. To be able to scientifically explain my contribution, I will briefly introduce the rationale and initial experiments.

To study the effects of sterile chronic inflammation on BM HSCs, we employed a mouse model of CMO (see "CMO mouse model" for details). First, we compared BM HSCs from CMO mice with those from wild-type (WT) controls. As anticipated, BM HSCs from CMO mice were significantly expanded—in both percentage and absolute numbers—accompanied by increased neutrophil counts and expansion of HSC downstream progenitors (Figure 6), consistent with the previous publications on sterile chronic inflammation and HSCs [163-165, 167, 168].

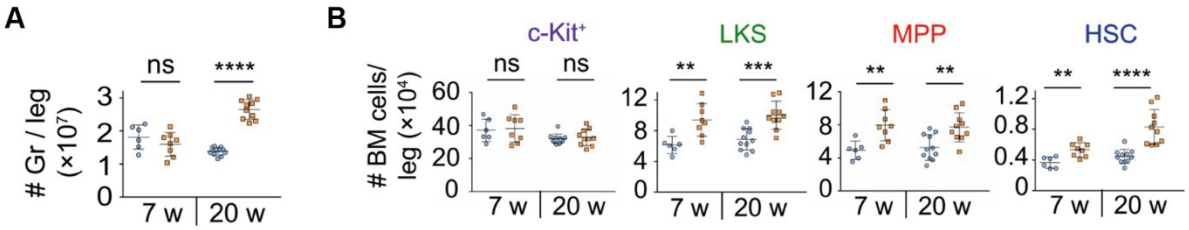


Figure 6: *(A) Total count of granulocytes in the BM. The Y-axis represents the number of cells per leg, while the X-axis shows the age of the mice (7 or 20 weeks); (B) Analysis of the absolute number of different stem and progenitor cell populations in the BM. Each data point corresponds to an individual biological replicate.*

We next assessed whether chronic inflammation affected HSC functionality. Extreme limiting dilution transplantation assays—a widely used technic to evaluate HSC function—revealed a significant reduction in the engraftment capacity of CMO BM HSCs compared to WT controls (Figure 7).

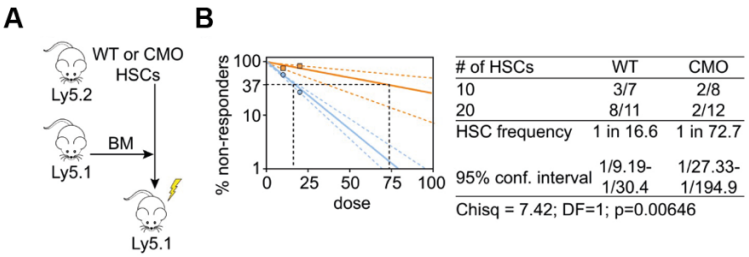


Figure 7: *(A) Schematic representation of the transplantation setup using 20-week-old WT and CMO HSCs; (B) Functional frequency of WT (blue) and CMO (orange) HSCs, assessed through limiting dilution competitive repopulation unit assays and calculated using ELDA online software based on Poisson distribution statistics. (Chi-square test; Chisq = 7.42; P = 0.00646).*

Previous studies have shown that IL-1β drives BM HSC proliferation and myeloid skewing via IL-1R/MyD88/NF-κB signaling [164, 248, 249]. To test whether IL-1β is solely responsible for HSPC expansion under sterile inflammation, we employed MyD88$^{flox/flox}$ VAV-iCre mice to delete MyD88 specifically in hematopoietic cells. Surprisingly, despite abrogation of IL-1 signaling in these cells, CMO MyD88$^{flox/flox}$ VAV-iCre+ mice still exhibited key features of the CMO phenotype—including swollen paws, deformed tail, an expanded HSPC compartment along with increased BM cellularity (Figure 8).

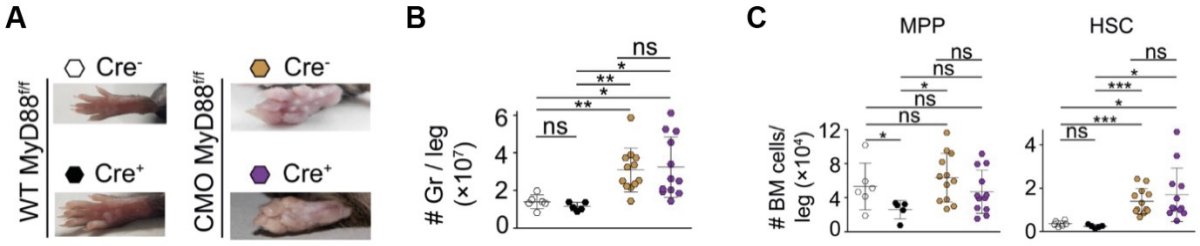


Figure 8: *(A) Representative images of paws from four mouse groups: WT MyD88$^{flox/flox}$ VAV-iCre^{-} (white symbol), WT MyD88$^{flox/flox}$ VAV-iCre^{+} (black symbol), CMO MyD88$^{flox/flox}$ VAV-iCre^{-} (brown symbol), and CMO MyD88$^{flox/flox}$ VAV-iCre^{+} (purple symbol); (B) Quantification of the absolute number of granulocytes per leg; (C) Quantification of the absolute number of MPPs and HSCs per leg. The Y-axis represents the number of MPPs (left panel) and HSCs (right panel) per leg.*

Since it was previously published that abrogation of the IL-1b/MyD88 signaling pathway was shown to rescue the inflammatory phenotype [143, 250], we hypothesize that the remaining

signaling in the non-hematopoietic compartment was mediating the phenotype. Therefore, we then crossed CMO mice with a whole body MyD88 knockout mice (KO). In these animals, the phenotypic signs of CMO (e.g., swollen paws) were absent, and BM cellularity, granulocyte expansion, and splenomegaly were prevented (Figure 9). These results were in agreement with the work of Dr. Lukens and Dr.Cassel [143, 250]. However, even without IL-1β/MyD88 signaling, the HSPC compartment remained expanded, and the functional impairment of BM MyD88 KO HSCs persisted compared to CMO controls (Figure 9 and data not shown).

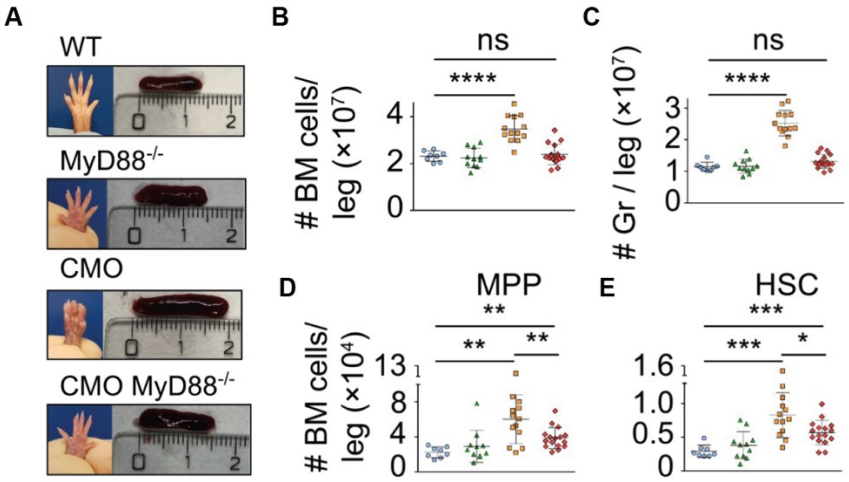


Figure 9: *(A) Representative images of paws and spleens from each experimental group; (B) Absolute number of BM cells per leg; (C) Quantification of the absolute number of granulocytes per leg; (D) Absolute number of MPPs and (E) HSCs per leg.*

Each symbol represents a 20-week-old mouse (biological replicate), with blue for WT, green for MyD88$^{-/-}$, orange for CMO, and red for CMO/MyD88$^{-/-}$ double-mutant mice.

These unexpected findings prompted us to investigate additional factors contributing to the detrimental effects on BM HSCs in CMO mice. To exclude any intrinsic influence of the *Pstpip2* point mutation on HSCs, we transplanted WT BM HSCs into either WT or CMO BM niches and performed bulk RNA sequencing on the transplanted cells. Among the differentially expressed genes, we observed a pronounced upregulation of a myeloid inflammatory signature, with significant enrichment of the IL-6/JAK/STAT3 signaling pathway (Figure 10). Notably, previous studies in CMO mice have reported that IL-6 is upregulated early in asymptomatic (6–8 week old) mice, while other pro-inflammatory cytokines become elevated later in symptomatic (20-week-old) mice [133]. These findings suggest that IL-6 may play a pivotal

role in initiating CMO disease, with the subsequent upregulation of additional cytokines correlating with disease progression.

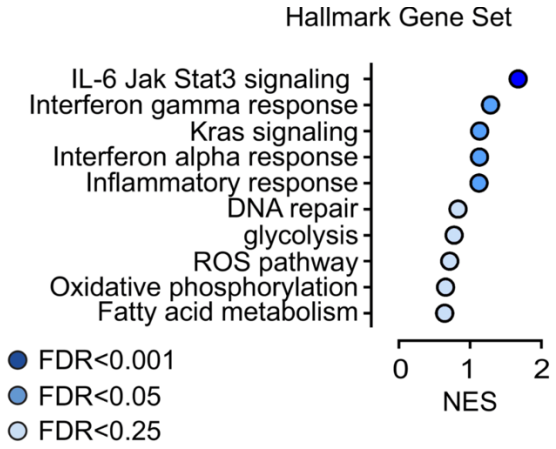


Figure 10: *Gene Set Enrichment Analysis (GSEA) highlighting the top relevant pathways that are upregulated in WT HSCs exposed to the CMO BM niche, compared to WT HSCs exposed to the WT BM niche. Data were generated using MSigBD Hallmark gene set v.7 (ranked according to NES values, FDR < 0.25).*

To further explore this hypothesis, we measured IL-6 levels via ELISA in BM fluid (cell-free supernatant) and serum. Symptomatic CMO mice exhibited significantly elevated IL-6 levels compared to healthy WT controls in both sample types (Figure 11). In parallel, we observed significant activation of pSTAT3, indicative of downstream IL-6 signaling, in BM HSCs from CMO mice.

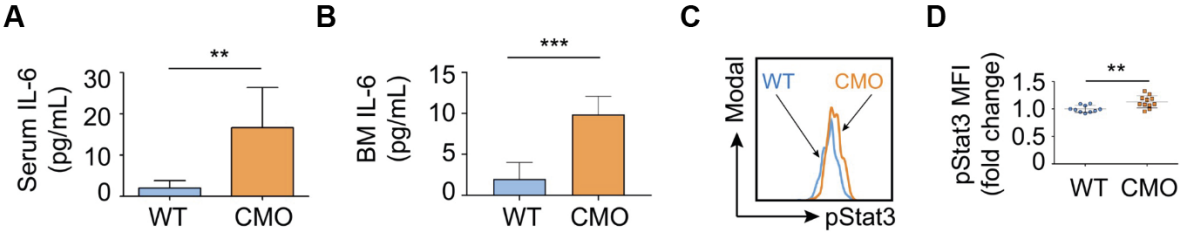
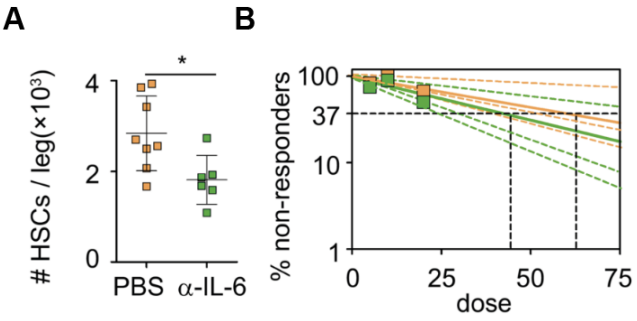


Figure 11: *(A) IL-6 levels in serum and (B) in BM fluid of WT (n = 6) and CMO (n = 8) symptomatic mice. The Y-axis represents IL-6 concentration (pg/ml); (C) Representative flow cytometry histograms showing pStat3 signal in WT (blue) and CMO (orange) HSCs; (D) Quantification of pStat3 signal from panel (C). The Y-axis represents pStat3 mean fluorescence intensity (MFI), normalized to the average WT value.*

To validate the role of IL-6/JAK/STAT3 signaling in driving HSC expansion, we first treated CMO mice with an IL-6 and IL-6 receptor (IL-6R) inhibitor. Although this treatment significantly reduced HSC numbers, the functional capacity of HSCs remained impaired relative to untreated CMO controls (Figure 12).

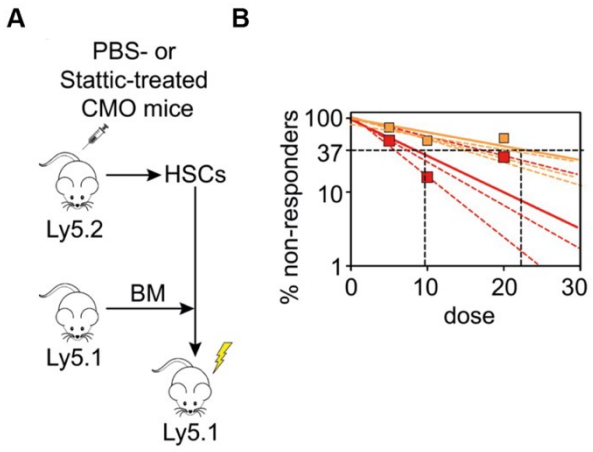


# of HSCs	PBS	α-IL-6
5	1/6	1/6
10	0/6	0/6
20	2/6	3/6
HSC frequency	1 in 62.6	1 in 44.3
95% conf. interval	1/20.5-1/193	1/17-1/117

Chisq = 0.21; DF=1; p=0.647

Figure 12: *(A) Absolute number of phenotypically identified HSCs per leg in CMO mice treated with PBS control (orange) or IL-6-blocking antibody (green); (B) Frequency of functional HSCs in the same treatment groups, assessed to evaluate the impact of IL-6 blockade.*

Consequently, we targeted downstream signaling by blocking STAT3 with the inhibitor Stattic, which resulted in an improvement in HSC functionality (Figure 13).



# of HSCs	PBS	Stattic
5	3/12	6/12
10	6/12	10/12
20	5/11	7/10
HSC frequency	1 in 22.1	1 in 9.69
95% conf. interval	1/13.05-1/37.7	1/6.31-1/15

Chisq = 6.09; DF=1; p=0.0136

Figure 13: *(A) Schematic representation of the experimental design; (B) Frequency of functional HSCs in CMO mice following treatment with Stattic (red) or PBS control (orange).*

As discussed in the section "The impact of chronic inflammation on HSCs," chronic inflammation in mice can be induced either experimentally or genetically, with reported effects on HSCs ranging from markedly detrimental to relatively mild [164-168]. In our study, we

employed a mouse model of progressive autoinflammatory disorder—CMO—which is based on a naturally occurring mutation that leads to sterile chronic inflammation [132, 139]. This model allowed us to bypass the artificial induction of inflammation and assess the consequences of a spontaneously developed inflammatory condition.

The inflammatory state of CMO is characterized by elevated levels of multiple pro-inflammatory cytokines, including MIP-1α, IL-1α, IL-1β, IL-6, IFNγ, MCP-1, G-CSF, and M-CSF, with hyperproduction of IL-1β which is predominantly associated with the CMO phenotype as a leading factor in disease development [133, 134, 136, 137, 143, 250]. In this study, we demonstrated that although IL-1β is widely recognized for its role in driving chronic inflammation and the CMO phenotype—as evidenced by the resolution of inflammatory symptoms in CMO MyD88 full-body KO mice—the expansion of HSPCs in CMO is predominantly driven by IL-6/JAK/STAT3 signaling rather than by IL-1β. Although there is strong evidence that chronic exposure of HSCs to IL-1β affects self-renewal [164, 249], IL-6, a multifunctional cytokine upregulated during inflammation and known as a potent driver of stress myelopoiesis [251, 252], appears to contribute to HSPC expansion in our model. Notably, IL-6 and IL-6R blockade alone did not fully rescue the functional defects observed in CMO BM HSCs; in contrast, inhibition of STAT3 improved HSC functionality, suggesting that additional cytokines that signal through STAT3 may also contribute to HSC expansion under sterile inflammatory conditions.

These findings imply that targeting STAT3 could modulate HSC biology and may offer a potential therapeutic strategy for treating CRMO and other patients suffering from autoinflammatory diseases.

3.2. EMH HSCs and Tregs cooperate to preserve extramedullary hematopoiesis under chronic inflammation.

This study represents my main PhD project, in which I explored the role of EMH HSCs under sterile chronic inflammatory conditions—a phenomenon that remains poorly understood. Here, I contributed to the design, performance, and analysis of all experiments. Currently, the paper is under revision in a peer-review journal, in which I am the first author. The manuscript is available in bioRxiv: https://doi.org/10.1101/2025.02.05.636492.

To begin, we confirmed the presence of circulating HSPCs and examined the spleen, the primary site of EMH, in our CMO mouse model of chronic inflammation. Our analyses revealed increased levels of HSPCs in both the circulation and the spleen (Figure 14), thereby confirming the presence of EMH—a finding that aligns with previous reports demonstrating that stress conditions favor EMH development.

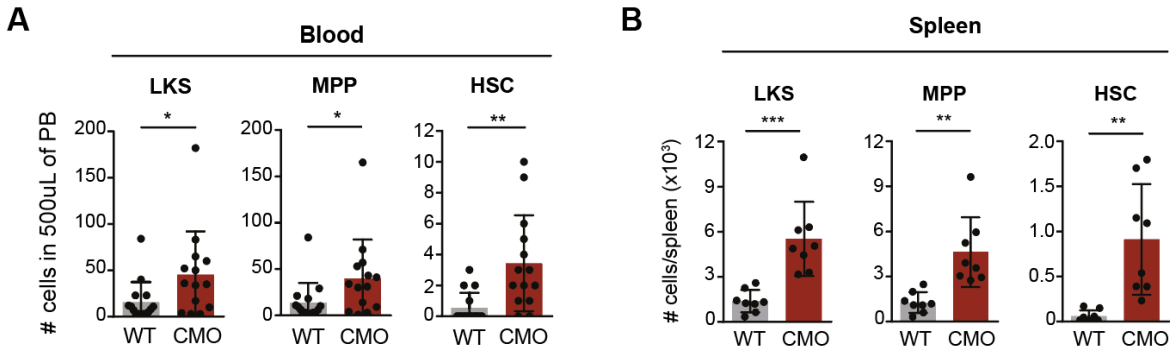


Figure 14: *Absolute number of LKS, MPP and HSC in blood (A) and spleens (B) of WT (gray) and CMO (red) mice.*

Furthermore, we performed transplantation experiments using cells isolated from the peripheral blood and spleen of both WT and CMO mice to verify the presence of true HSCs, defined by their ability to engraft into recipient mice and sustain hematopoietic reconstitution. In both cases, HSPCs from CMO mice exhibited higher engraftment than WT controls, suggesting that tissues from CMO mice harbor a greater number of functional HSCs (Figure 15).

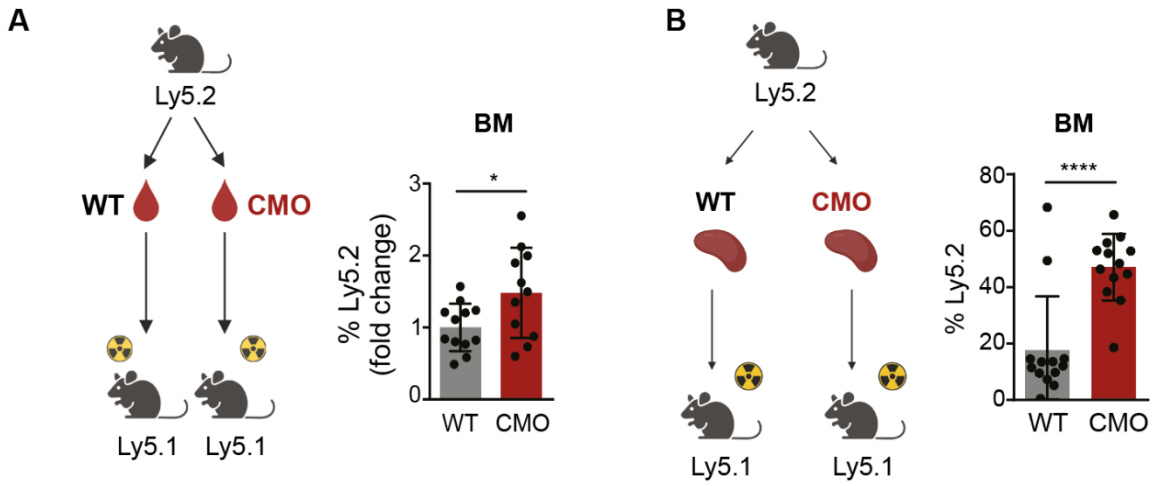


Figure 15: *(A) Schematic representation of the WT (grey) and CMO (red) whole blood transplantation into lethally irradiated recipients, followed by engraftment evaluation 16 weeks post-transplantation in the BM; (B) Schematic representation of the WT (grey) and CMO (red) splenocyte transplantation into lethally irradiated with engraftment assessed 16 weeks post-transplantation in the BM.*

Since the spleen is a well-established site of EMH, we sought to determine whether our CMO mice develop additional, atypical sites of EMH. In a study by Regan-Komito et al., a mouse model of experimental spondyloarthritis showed ongoing EMH in inflamed paws, as was confirmed by colony culture assays in which only hematopoietic progenitors were capable of growing and differentiating into colonies [167]. Inspired by these findings, we investigated whether our CMO mice also harbor HSPCs in the inflamed paws. Using flow cytometry, we phenotypically identified HSPCs in the inflamed paws of CMO mice, whereas WT paws showed virtually no HSPCs (Figure 16).

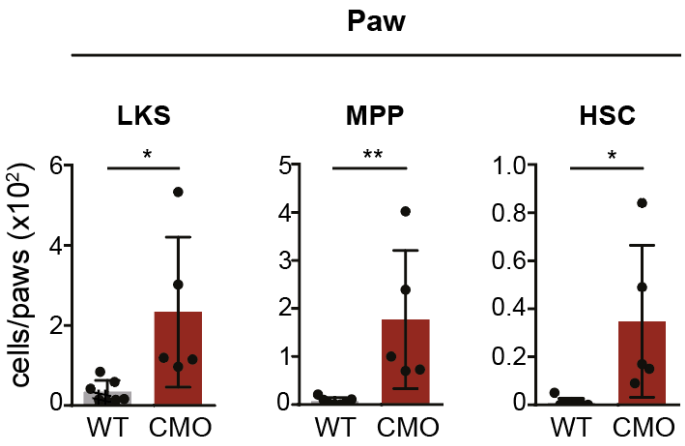


Figure 16: *Absolute number of LKS, MPP and HSC in the hind paws of WT (gray) and CMO (red) mice.*

To confirm that these cells were indeed functional HSCs, we performed extreme limiting dilution transplantation assays by transplanting HSCs isolated from CMO paws into lethally irradiated recipients and comparing their repopulating capacity to that of WT BM HSCs. Strikingly, we observed no significant differences in engraftment between WT BM HSCs and CMO paw HSCs, indicating that the inflamed paws of CMO mice harbor functional HSCs of similar quality than the WT HSCs (Figure 17).

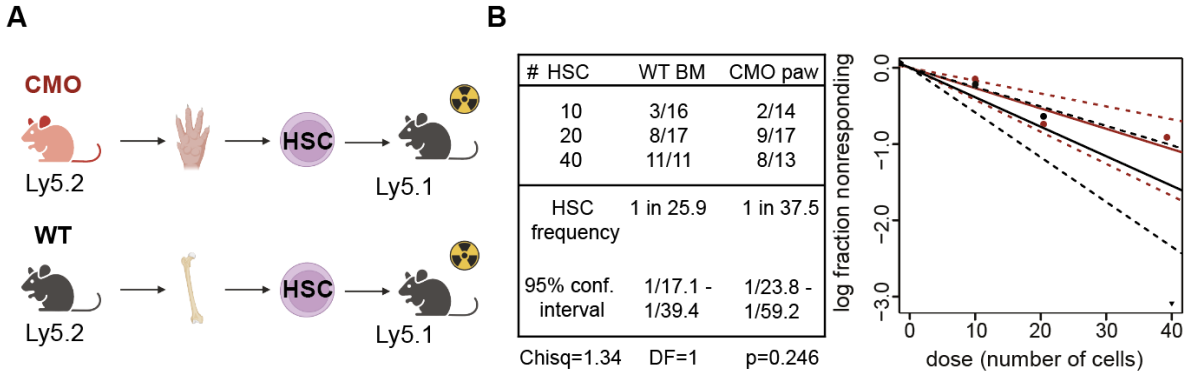


Figure 17: *(A) Schematic representation of the WT BM HSC (black) and CMO paw HSC (red) transplantation into lethally irradiated recipients; (B) Frequency of functional HSCs from CMO paw and WT BM, assessed using limiting dilution competitive repopulation unit assays. Frequencies were calculated using ELDA online software based on Poisson distribution statistics.*

To elucidate the biological role of EMH HSCs and how they differ from WT and CMO BM HSCs, we performed single-cell sort-sequencing on cells isolated from these tissues (Figure

18). Unsupervised clustering revealed that cells from CMO paws and spleen predominantly formed a distinct cluster, named as cluster 3.

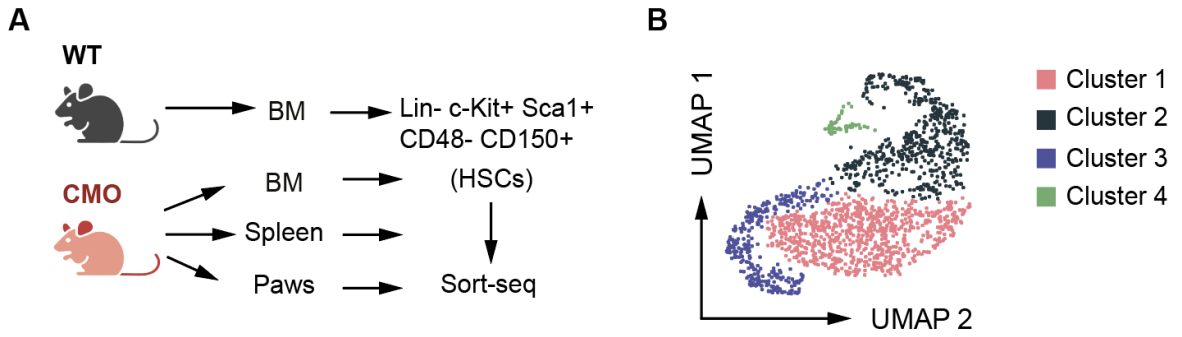


Figure 18: *(A) Graphical representation illustrating the isolation and sorting of HSCs from various sources. HSCs were identified as Lin⁻ c-Kit⁺ Sca-1⁺ CD48⁻ CD150⁺ cells and subsequently sorted for sort-sequencing analysis; (B) UMAP plot of HSC transcriptomes showing four distinct clusters identified through unsupervised clustering.*

Differential expression analysis of this cluster identified *Cd53*—a tetraspanin uniquely expressed in hematopoietic cells—as one of the most significantly upregulated genes (Figure 19).

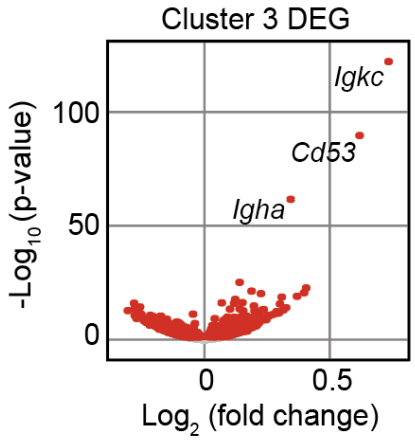


Figure 19: *Volcano plot highlighting the most differentially expressed genes in cluster 3 compared to the other identified clusters.*

Although CD53 has been extensively studied in mature blood cells, where it was shown to participate in T- and NK cell signaling, neutrophil migration, and B cell development [253-259], only two reports have addressed its role in HSCs. One study suggested that CD53+ HSCs are more quiescent [260], while another found that splenic HSCs upregulate CD53 in response to stress to suppress proliferation [261]. Based on these findings, we aimed to further explore the role of CD53 in EMH HSCs. First, we confirmed that CD53 protein levels are increased in

HSCs isolated from extramedullary sites (spleen and paws) compared to BM HSCs (Figure 20).

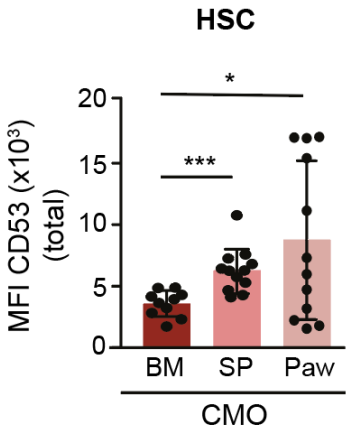


Figure 20: *Total CD53 levels in HSCs isolated from BM, SP, and paw of CMO mice. The Y-axis represents CD53 MFI.*

However, not all EMH HSCs expressed CD53 on their surface, allowing us to subdivide them into CD53+ and CD53- populations. We then compared the functionality of splenic CD53+ and CD53- HSCs using transplantation assays and found that the CD53+ HSCs exhibited enhanced functionality (Figure 21). These results align with previous reports suggesting that CD53+ HSCs are more quiescent and possess long-term repopulating potential.

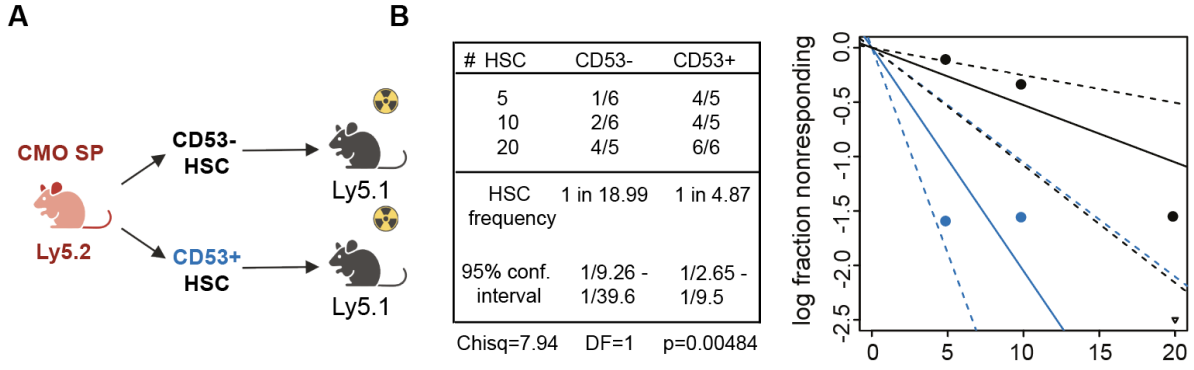


Figure 21: *(A) Schematic representation of the transplantation setup. CD53⁻ or CD53⁺ HSCs (5, 10, or 20 cells) isolated from CMO SP were transplanted into lethally irradiated congenic mice. Recipients were sacrificed and analyzed 16 weeks post-transplantation; (B) Frequency of functional CD53⁻ and CD53⁺ HSCs from CMO SP, assessed using limiting dilution competitive repopulation unit assays. Frequencies were calculated using ELDA online software based on Poisson distribution statistics.*

It was previously demonstrated that CD53 is found in close proximity to MHC class II on the plasma membranes of B cells and DCs [262-264]. Accordingly, we examined the expression

of MHC class II-related genes in cluster 3 and observed a notable upregulation of both MHCII-related and immunoregulatory genes (Figure 22).

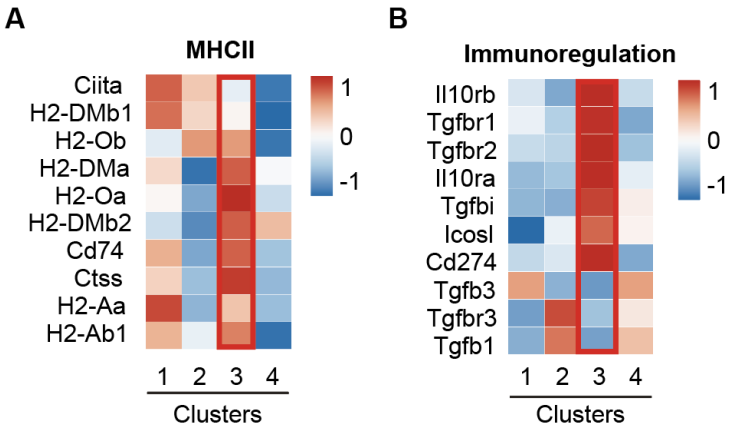


Figure 22: *(A) Relative expression of MHCII-associated genes across distinct HSC clusters; (B) Relative expression of immunoregulation-associated genes across distinct HSC clusters. The red box highlights cluster 3, which consists of EMH HSCs.*

Based on these findings, we hypothesized that CD53+ EMH HSPCs might interact with T cells. To test whether CD53+ HSPCs can activate naïve T cells, we employed an OVA peptide system along with naïve T cells isolated from Foxp3 reporter mice, whose TCRs specifically recognize OVA. We then incubated both CD53+ and CD53- HSPCs isolated from the spleen with naïve T cells for four days and subsequently evaluated T cell proliferation, viability, and Treg induction. Interestingly, CD53+ HSPCs efficiently activated naïve T cells, enhancing their viability and proliferation, whereas CD53- HSPCs induced T cell anergy, as indicated by increased CD73 expression (Figure 23). Moreover, CD53+ HSPCs promoted the development of Tregs, as evidenced by Foxp3-GFP expression. In terms of HSPCs, we observed that after four days of incubation, CD53+ HSPC cultures contained c-kit+ cells, whereas CD53- HSPC cultures did not (Figure 23).

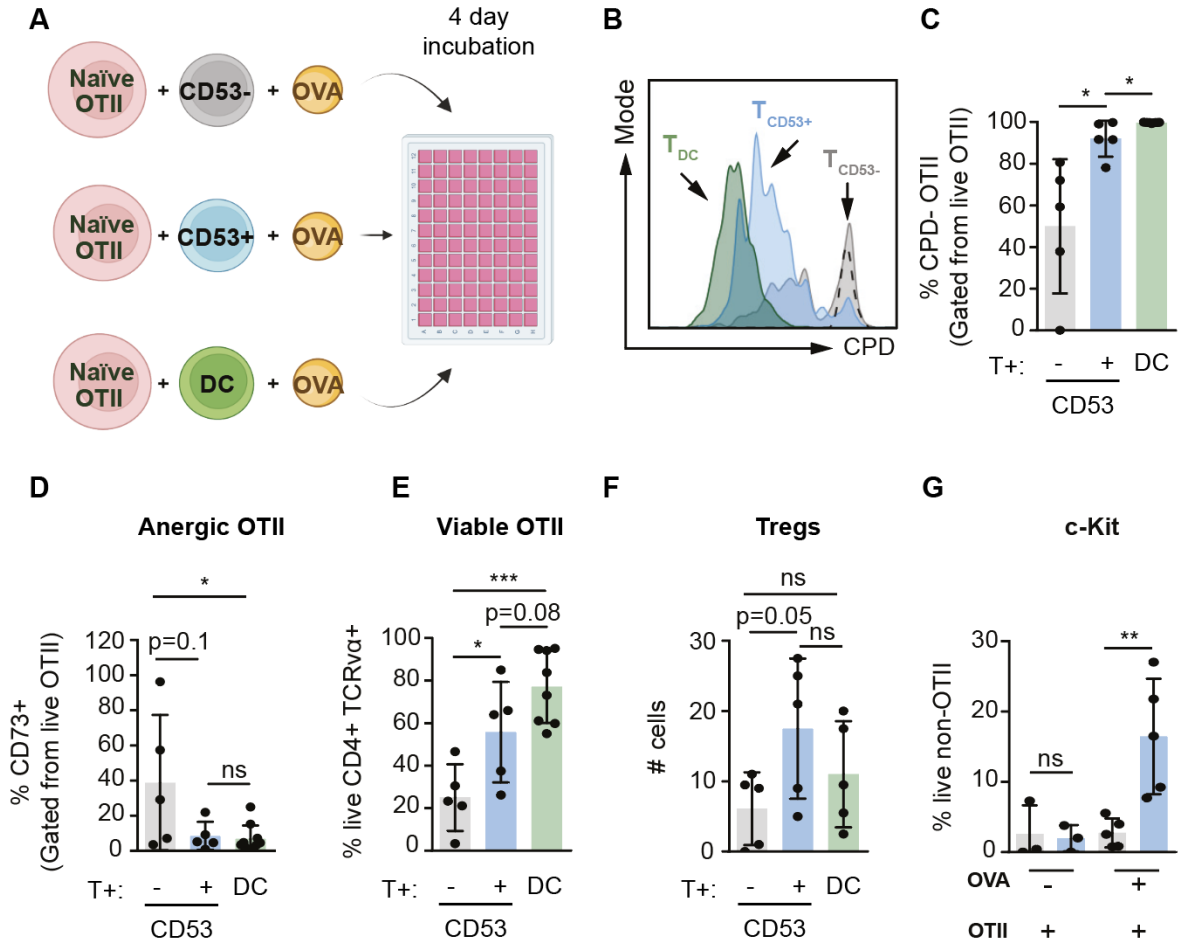


Figure 23: *(A) Schematic representation of co-culture experimental setup used to assess interactions between HSPCs and naïve OT-II cells in the presence of OVA 247-264 peptide; (B) Representative histogram plot showing T cell proliferation after 4 days in co-culture with dendritic cells (T_{DC}; green), CD53⁺ HSPCs (T_{CD53^+}; blue), or CD53⁻ HSPCs (T_{CD53^-}; gray); (C) Frequency of proliferated CD4⁺ OT-II T cells after co-culture; (D) Frequency of anergic CD4⁺ OT-II T cells after 4 days of co-culture; (E) Frequency of viable CD4⁺ OT-II T cells after co-culture; (F) Number of OT-II Tregs after co-culture measured by Foxp3-GFP expression; (G) Frequency of c-Kit⁺ HSPCs after co-culture with naïve CD4⁺ OT-II cells. The first two columns represent negative control co-cultures containing HSPCs and naïve CD4⁺ OT-II cells without OVA. The remaining columns represent co-cultures with HSPCs, naïve CD4⁺ OT-II cells, and OVA. HSPCs were either CD53⁻ (gray) or CD53⁺ (blue).*

Altogether, these findings led us to hypothesize that EMH HSCs may upregulate CD53 to promote Treg development and thereby preserve the HSC pool. To test whether HSCs are affected by Treg ablation, we treated mice with an anti-CD25 antibody, a commonly used reagent for depleting Tregs [265-268]. After one month of treatment, we evaluated HSC proliferation as well as CD53 and MHCII expression on HSCs. As expected, Treg depletion resulted in increased HSC proliferation, interestingly it was accompanied by a decrease in both CD53 and MHCII expression (Figure 24).

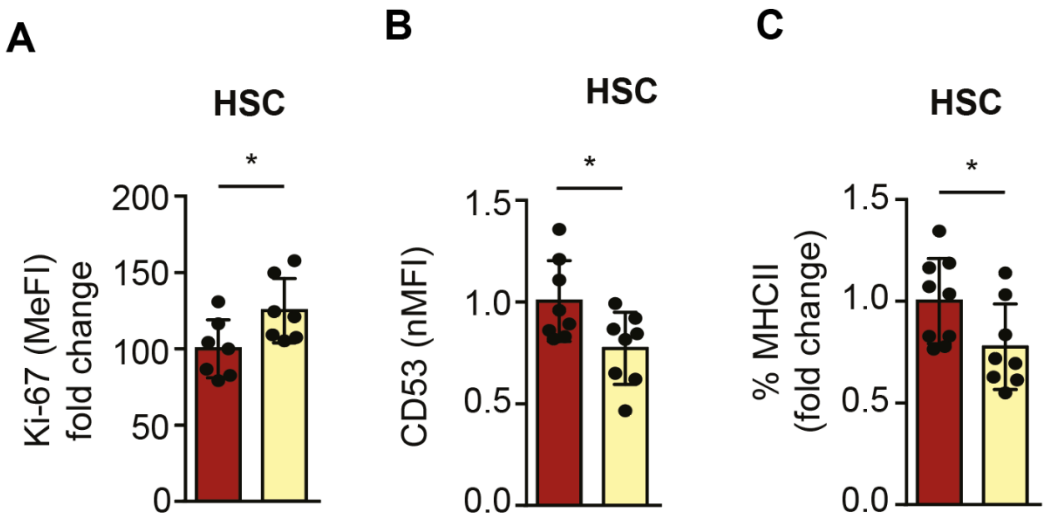


Figure 24: *(A) Ki-67 MFI in SP HSCs from CMO control non-treated mice (red) and CMO a-CD25-treated mice (yellow); (B) Flow cytometric analysis of total CD53 expression in SP HSCs from CMO mice. CD53 expression is shown as a fold change relative to the non-treated CMO group (red). The Y-axis represents normalized mean fluorescence intensity (nMFI); (C) Frequency of MHCII⁺ HSCs in the SP of CMO mice.*

Although stress conditions are known to induce EMH, the precise role of these HSCs remains largely unclear. Our study reveals a novel function for EMH HSCs under chronic inflammatory conditions where they upregulate CD53 and MHCII-related molecules as a mechanism to preserve EMH in peripheral tissues. This upregulation may represent an adaptive response by a subpopulation of EMH HSCs—those expressing CD53—to suppress inflammation rather than promote it by supporting myelopoiesis as seen in cancer.

CD53 has been extensively characterized in mature blood cells, however its role in HSC biology remains elusive. Our results demonstrate that CD53⁺ HSCs can efficiently activate naïve T cells and promote Treg development *in vitro*. Similarly, a CD53⁺ megakaryocyte population emerging under inflammatory conditions shows high efficiency in activating naïve

T cells [269], paralleling our findings with EMH $CD53^+$ HSCs. Notably, our single-cell data did not reveal the expression of classical co-stimulatory molecules, such as CD80 and CD86, in EMH HSPCs, raising additional questions about how $CD53^+$ EMH HSCs activate naïve T cells. Moreover, $CD53^+$ DCs exhibited similar enhanced activation of naïve T cells compared to their $CD53^-$ counterparts (data not shown), suggesting that CD53 expression on the cell surface may generally enhance T cell activation.

Previous studies have demonstrated that Tregs exert a protective effect on BM HSCs by modulating their survival and promoting clonal expansion [52, 54]. In our work, we showed that depletion of Tregs resulted in increased proliferation of EMH HSCs, which, in turn, led to reduced functionality and a shift toward myeloid priming, as evidenced by transplantation assays. These results indicate that Treg presence is essential for maintaining a low proliferative state in EMH HSCs thereby preserving their functional integrity.

It is both intriguing and fascinating that cells not traditionally associated with antigen presentation and T cell activation can adapt these functions under stress conditions, for reasons that remain to be fully understood. Although antigen presentation is not a common feature of HSCs, several studies have detected MHCII expression on their surface, albeit with varying interpretations of its significance [51, 53, 54]. Further research is needed to elucidate the role of MHCII in both steady-state and stress conditions, as well as to clarify the role of CD53 in antigen presentation, the mechanisms by which it regulates T cell activation, and how its expression is controlled on the cell surface.

3.3. The genetic background affects neutrophil activity and determines the severity of autoinflammatory osteomyelitis in mice.

Since murine models are essential for basic research—and many genes and proteins are initially characterized in mice before being validated in humans—it is crucial to understand how the genetic background of mice influences experimental outcomes, particularly in studies investigating immune responses and disease mechanisms. In this section, I will present our results published in the Journal of Leukocyte Biology. I will introduce the main results of the project and present my contribution in Figures 25A, 26, 28.

Three of the most common murine strains used in research are BALB/c, C57BL/6J, and C57BL/6NCrl (also referred to as C57BL/6N). It is well established that in terms of adaptive immune response BALB/c mice tend to exhibit a type 2 immune response, whereas C57BL/6J mice are biased toward a type 1 (Th1) response, which contributes to their higher resistance to *Listeria monocytogenes* infection [270-274]. Although the genetic differences among these strains are relatively well characterized, significant gaps remain in our understanding of their phenotypic variations in innate immunity. Only a few studies have compared the immune responses between C57BL/6J and C57BL/6N mice, describing sex-specific variations in susceptibility to *L. monocytogenes* [275], differences in contact hypersensitivity [275], lupus-induced nephritis [276], influenza A virus-mediated inflammatory disease [277], brain inflammation in response to poly(I:C) [278], and neutrophil recruitment to the lung during inflammatory challenges [279]. However to date, there is currently no comprehensive analysis comparing the innate immune responses of BALB/c, C57BL/6J, and C57BL/6N mice.
Given that our CMO mice develop sterile chronic inflammation driven primarily by the innate immune system (as evidenced by the recovery observed upon neutrophil depletion), this model provides a unique platform to evaluate potential differences in innate immune responses across various genetic backgrounds. Accordingly, we derived the *Pstpip2* mutation into three murine strains, enabling us to investigate how genetic variability influences the development of sterile chronic inflammation and the corresponding innate immune responses within the CMO model.

One of the first differences we observed among the strains was the timing of symptom onset (Figure 25). Although all strains eventually developed osteomyelitis, the disease onset was significantly delayed in BALB/c and C57BL/6J mice compared to C57BL/6N mice. These

results were further confirmed by microtomography, which revealed milder bone damage and less pronounced tissue swelling in BALB/c and C57BL/6J mice relative to C57BL/6N mice.

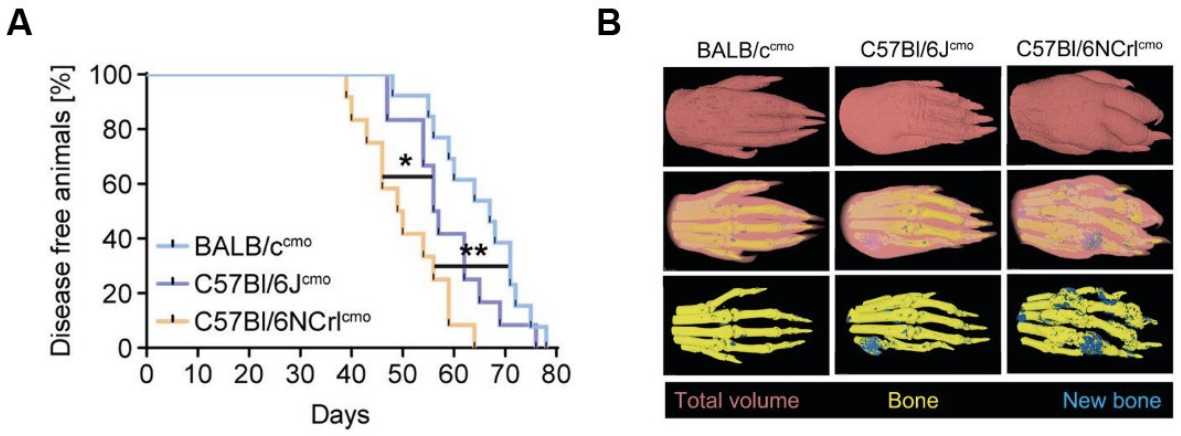


Figure 25: *(A) Comparison of disease progression across three CMO genetic backgrounds: BALB/c, C57BL/6J, and C57BL/6NCrl mice; (B) Representative µCT reconstructions of the paws from 10-week-old CMO females.*

Since osteomyelitis is strongly associated with myeloid cell infiltration at the site of inflammation [133, 136, 139, 141], we further quantified the numbers of myeloid cells in the hind paws. In line with the observed severity of disease symptoms in CMO mice, we found that the C57BL/6N strain exhibited significantly higher numbers of both neutrophils and monocytes in their paws compared to C57BL/6J and BALB/c mice (Figure 26). This observation correlates with the earlier disease onset and more pronounced bone degradation observed in the C57BL/6N strain. Surprisingly we did not observe changes in IL-1β levels between the strains which might explain that disease developed in all the strains but apparently does not have an effect on disease severity.

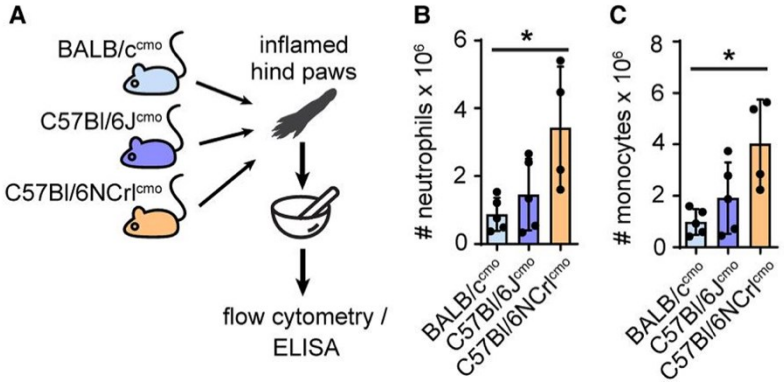


Figure 26: *(A) Schematic representation of CMO paw processing and subsequent sample analysis; (B) Absolute counts of Ly6G+ neutrophils and (C) Ly6C+ monocytes in hind paws of CMO mice from three genetic backgrounds.*

Since neutrophil migration to the site of inflammation depends on chemokines, we assessed the levels of key chemokines in the hind paws—the primary site of inflammation—to determine whether there were differences among the three genetic backgrounds. Hindfoot lysates were analyzed for CCL3, CXCL1, and CXCL2. C57BL/6N mice exhibited significantly higher levels of CCL3 and CXCL2, while only CXCL2 levels were significantly elevated in C57BL/6J mice compared to BALB/c mice (Figure 27). No statistically significant differences in CXCL1 concentrations were observed among the strains.

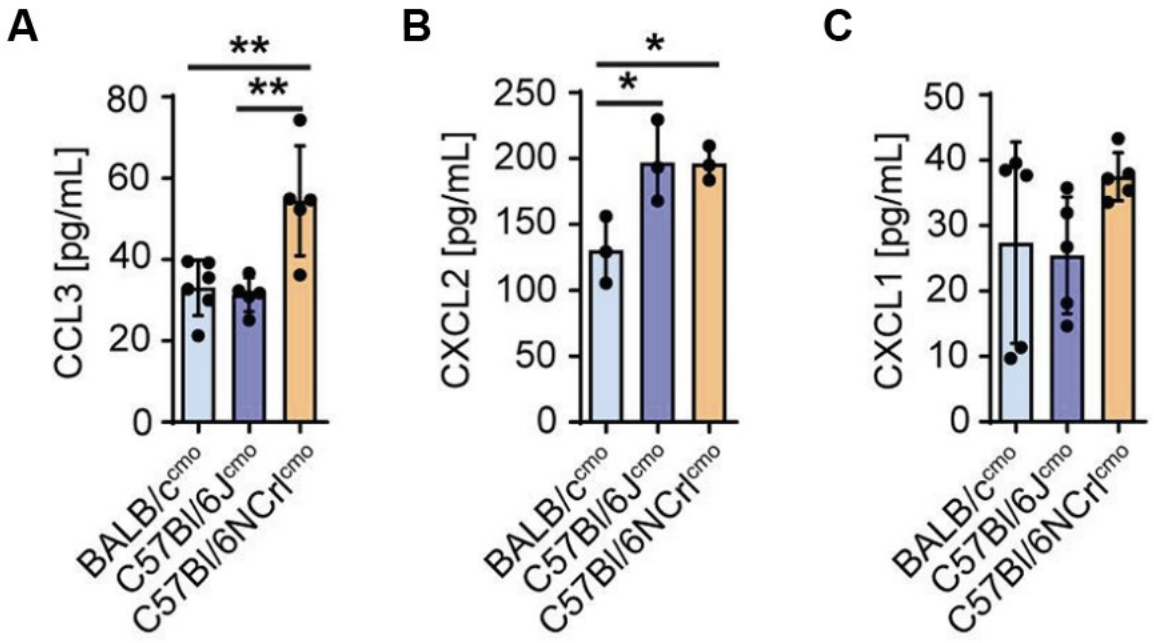


Figure 27: *(A), (B) and (C) show chemokine levels in hind paw lysates, measured by ELISA. Each dot represents the lysate of one paw.*

We also evaluated the migratory abilities of CMO BM neutrophils isolated from all three strains toward CCL3 and CXCL2, given the significant differences observed in the levels of these chemokines. Although some trends were noted, no statistically significant differences in neutrophil migration were detected among the strains (Figure 28).

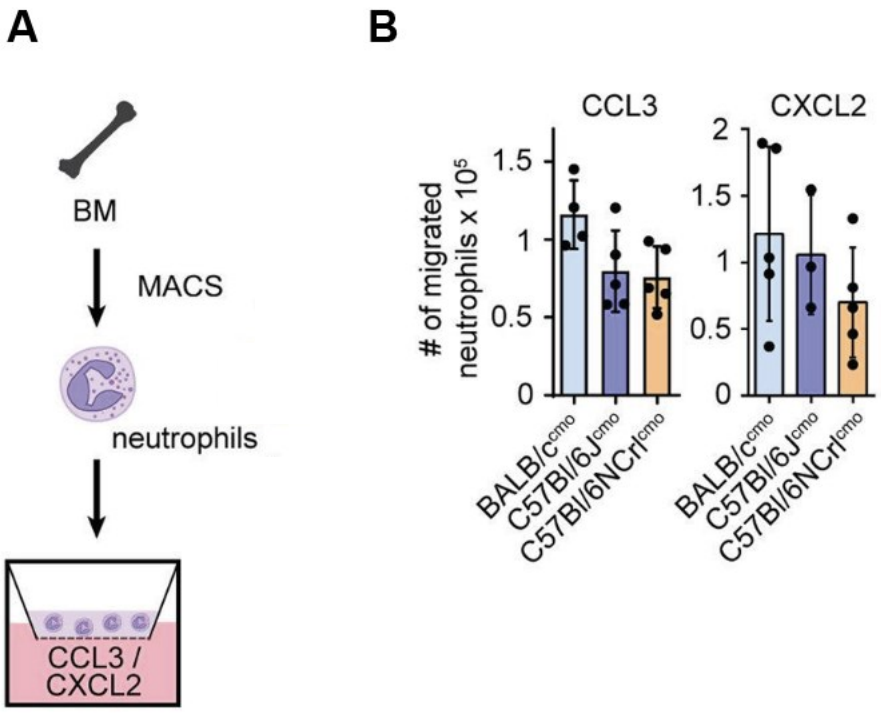


Figure 28: *(A) Schematic representation of the experimental setup for the Transwell assay, illustrating the process of neutrophil isolation from CMO BM and their subsequent migration towards CCL3 and CXCL2; (B) Absolute counts of neutrophils from BALB/c, C57BL/6J, and C57BL/6N CMO mice that migrated toward CCL3 or CXCL2 in the Transwell assay.*

Once activated, neutrophils can cause collateral damage to tissues at sites of migration or residence; therefore, their activation is tightly regulated. One of the regulatory mechanisms is mediated by inhibitory receptors bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), such as Ly49Q, PIR-B, SIRP-α, and Siglecs [280]. These receptors modulate key neutrophil functions—including migration, proliferation, inflammatory cytokine production, adhesion, and reactive oxygen species production [281, 282]. PIR-B and SIRP-α, in particular, are ubiquitously expressed on the surface of neutrophils and are also stored in their granules [283].

Importantly, the BALB/c strain, which exhibits the lowest disease severity, demonstrated the highest levels of these inhibitory receptors on neutrophils. Similarly, neutrophils isolated from the paws of WT BALB/c mice showed elevated expression of inhibitory receptors, with a statistically significant increase observed for PIR-B (Figure 29). These findings suggest that inhibitory receptors play a critical role in controlling neutrophil-mediated tissue damage and that their higher expression may contribute to the reduced disease severity observed in BALB/c mice.

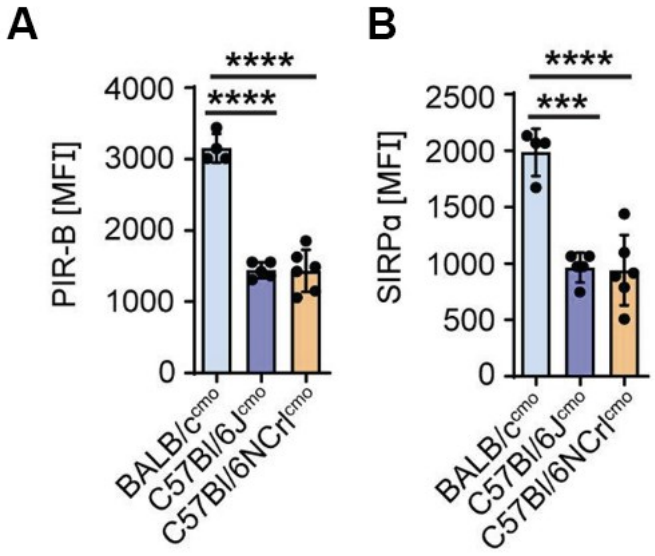


Figure 29: *MFI of PIR-B (A) and SIRP-α (B) on CMO paw neutrophils from three genetic backgrounds.*

Given the critical role of neutrophils in CMO disease development and the influence of genetic background on neutrophil biology, we isolated neutrophils from all three CMO strains and performed bulk RNA sequencing to identify genes that might underlie genetic differences in disease progression. Enrichment analysis of differentially expressed genes revealed significant overrepresentation of transcripts associated with rheumatoid arthritis, autoimmune diseases, and inflammation-related signaling pathways (Figure 30). Notably, nearly all of these genes were downregulated in BALB/c mice—the strain with the mildest disease manifestations—including *Padi4* and *Stat4*, which have been linked to rheumatoid arthritis in humans [284-287], and Ym1-Chil3, associated with collagen-induced arthritis and other immunopathologies in mouse models [288].

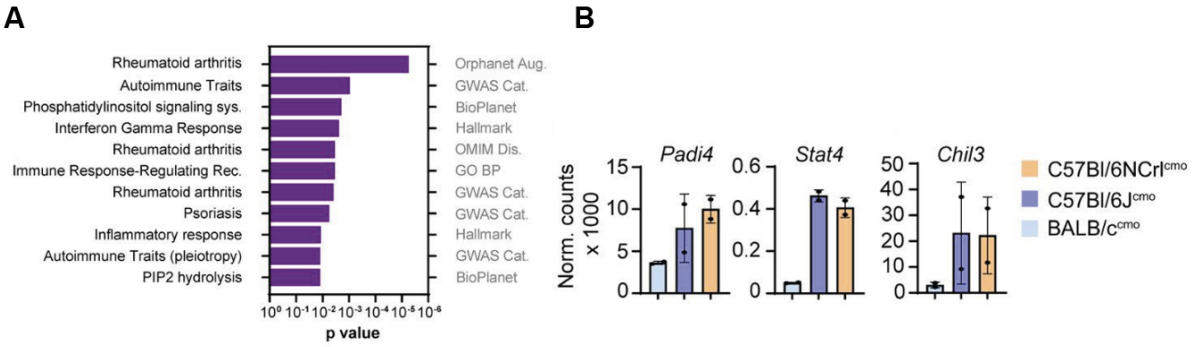


Figure 30: *(A) Relevant results of enrichment analysis of differentially expressed genes using Enrichr, highlighting significantly enriched pathways; (B) Expression of RA-related genes (Padi4, Stat4, and Chil3) presented as normalized counts, illustrating their differential expression across the analyzed CMO strains.*

Additionally, we detected several pathways that were downregulated in BALB/c mice, potentially contributing to the observed differences in inflammatory responses. These include the phosphatidylinositol signaling pathway (e.g., *Pik3c3*), inflammatory response pathways (e.g., *IL15*, *Ahr*, *Eif2ak2*, and *CD69*), and PIP2 hydrolysis (e.g., *Dgkh* and *Itpr1*) (Figure 31). Furthermore, distinct strain-specific expression patterns were observed in genes associated with neutrophil activation and degranulation. In BALB/c mice, lower expression of these gene markers suggests that neutrophil degranulation and activation are attenuated, indicating that genetic background modulates key processes governing neutrophil function in the context of CMO.

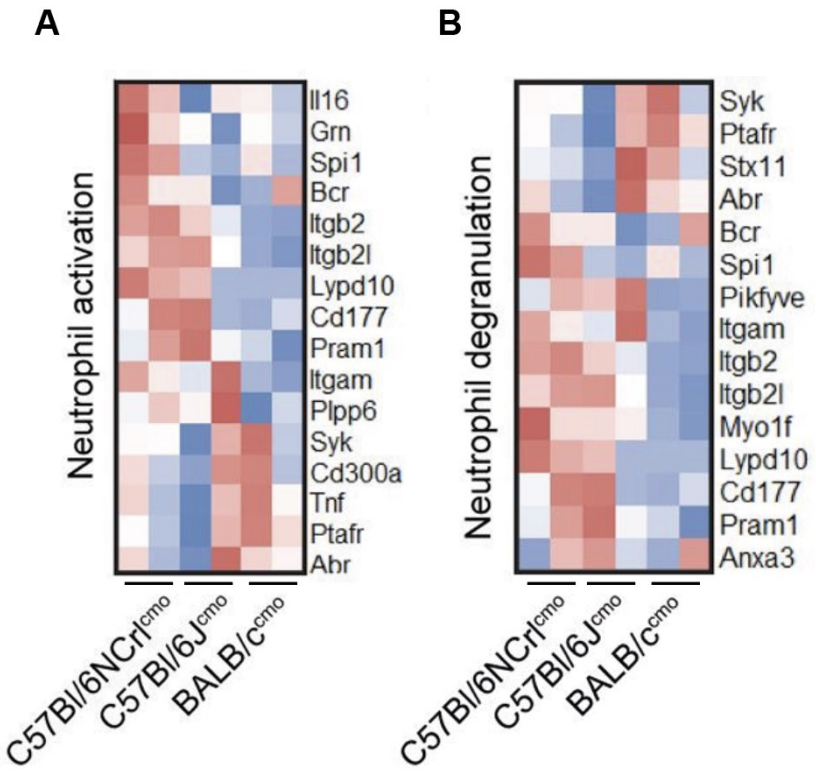


Figure 31: *(A) Expression of genes from the MSigDB ontology "GOBP: Regulation of Neutrophil Activation" and (B) "GOBP: Neutrophil Degranulation" across different CMO strains, highlighting strain-specific differences in gene expression.*

To verify the observed decrease in neutrophil degranulation in BALB/c mice, we measured key markers of neutrophil activation, including CD11b and CD62L (shedding), as well as CD63, a specific marker for degranulation [289, 290]. We then compared these markers across WT strains. Consistent with our transcriptomic analysis, BALB/c mice exhibited attenuated neutrophil activation and degranulation compared to C57BL/6 mice (Figure 32). In contrast, no significant differences in activation or degranulation were observed among the WT strains, indicating that genetic background markedly influences neutrophil activation and degranulation in the context of sterile chronic inflammation, while its impact on healthy WT mice is negligible.

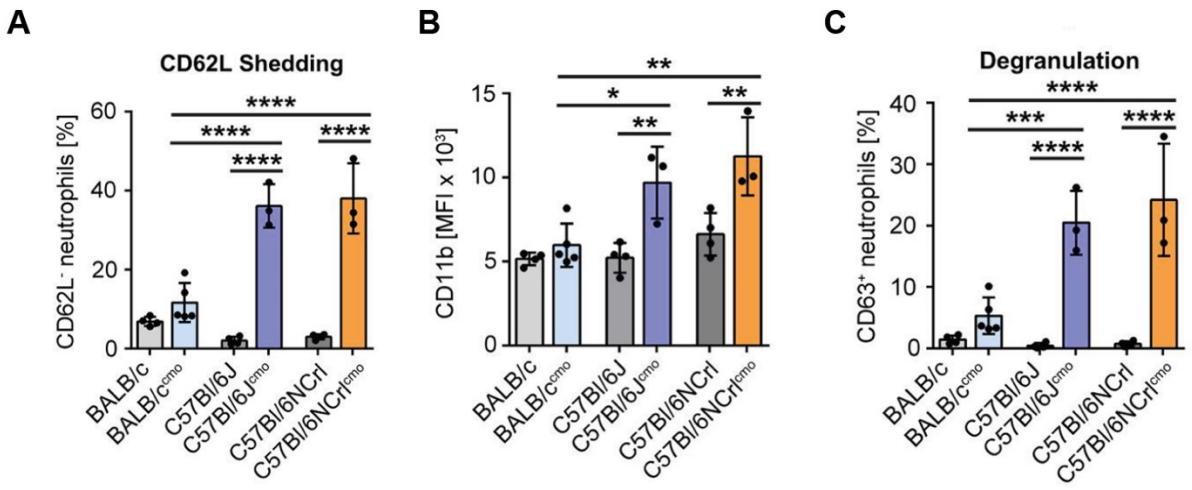


Figure 32: *(A) Percentage of CD62L⁻ neutrophils, (B) MF) for CD11b and (C) percentage of CD63⁺ neutrophils, illustrating neutrophil activation and surface marker expression across the analyzed conditions.*

Taken together, this study provides valuable insight into how innate immune responses vary among three of the most commonly used mouse strains in the context of autoinflammatory disease. We demonstrated that although all three strains develop chronic sterile inflammation, the onset and severity of disease differs significantly and correlates with differences in neutrophil biology—reflected in the levels of inhibitory receptors, neutrophil activation and degranulation, and distinct transcriptomic profiles. Overall, our data indicate that these strains are not interchangeable for studies of autoinflammatory diseases, as genetic background markedly influences innate immune responses and disease outcomes.

4. CONCLUSIONS

Chronic inflammation poses a significant threat to human health by establishing a persistent, low-grade inflammatory state that disrupts cellular functions [126]. It can arise either from autoimmune/autoinflammatory diseases or naturally during aging, phenomena termed as "inflammaging" [291]. This sterile chronic inflammation, driven by internal stimuli rather than external pathogens, has been implicated in the pathogenesis of various pathological conditions—including type 2 diabetes [292], cardiovascular disease [293], cognitive impairment [294], and brain atrophy [295]. Thus, elevated levels of pro-inflammatory factors play a critical role in the development of these diseases. Nevertheless, so far it has been challenging to determine what is the cause and what is in fact the consequence, since these pathologies also mediate the upregulation of pro-inflammatory cytokines.

It is increasingly evident that long-term, low-grade inflammation also impacts the function of various cell types, including HSCs, which lie at the apex of the hematopoietic hierarchy. Although numerous studies have focused on the effects of acute inflammation on HSCs [296-304], the influence of sterile chronic inflammation on these cells remains relatively unexplored. Given the essential role of hematopoiesis in sustaining blood cell production and ensuring proper immune responses, understanding how chronic inflammation alters HSC function is of paramount importance. We can hypothesize that systemic chronic inflammation may (1) promote HSC aging, (2) induce the development of clonal hematopoiesis, and (3) enhance the transformation from a pre-leukemic state to a full-blown leukemia. Recent studies from our laboratory and others suggest that indeed chronic inflammation can influence all these aspects of hematopoiesis, which opens the question of early intervention of chronic inflammation in clinics. We envision that therapeutic approaches to modulate chronic inflammation before HSC aging, clonal hematopoiesis, and leukemia may have a beneficial outcome either preventing or delaying these conditions.

In our laboratory, we focused on how sterile chronic inflammation affects HSC biology to gain insights into potential targeting strategies. To investigate this, we employed a mouse model of chronic multifocal osteomyelitis (CMO). In this model, sterile chronic inflammation develops gradually as the mice age, driven by a point mutation in the *Pstpip2* gene—a negative regulator of myeloid cell activation [132-134, 136, 137, 139, 305]. Similar to human autoinflammatory

diseases and aging, CMO mice exhibit overactivation of the innate immune system, with elevated levels of pro-inflammatory cytokines and chemokines, and display bone damage in the hind paws and tail. Importantly, PSTPIP2 is expressed in mature myeloid cells (e.g., macrophages, neutrophils, mast cells, and osteoclasts) but is absent in immature cells such as HSPCs, making the CMO model particularly suitable for studying the effects of chronic inflammation on HSCs [133].

In our CMO model, elevated levels of IL-1β have been reported, and previous studies have linked chronic exposure to IL-1β with HSC expansion, reduced functionality, and the activation of myeloid gene programs [164, 249]. Consistent with these reports, we observed an expansion of both HSCs and myeloid cells, along with reduced HSC functionality as demonstrated by transplantation assays. However, depletion of the MyD88 adaptor protein in CMO HSCs did not prevent their expansion, suggesting that IL-1β may not be the sole driver of these effects. Instead, we observed a marked upregulation of the IL-6/JAK/STAT3 pathway in HSCs, which appears to drive their proliferation and functional decline under conditions of sterile chronic inflammation. Given that IL-6/JAK/STAT3 signaling is known to activate genes involved in anti-apoptosis, survival, and proliferation [306-309]—processes well established in cancer—we hypothesized that this pathway plays a similar role in HSCs during sterile chronic inflammation. While blockade of IL-6 alone only modestly reduced HSC expansion, inhibition of STAT3 significantly improved HSC functionality, suggesting that additional cytokines signaling through the JAK/STAT3 pathway contribute to both the expansion and impaired function of HSCs. Interestingly, studies in rheumatoid arthritis models have shown that whole-body conditional STAT3 KO mice are significantly resistant to collagen-induced arthritis [310]—a model that shares similarities with CMO. These findings imply that targeting STAT3 may offer a promising therapeutic strategy for reducing HSC expansion, improving their functional properties, and mitigating overall inflammation in autoinflammatory and autoimmune diseases.

Currently, STAT3 inhibitors are primarily utilized in clinical settings for cancer treatment due to the role of STAT3 in tumor growth, metastasis, and immunosuppression. Several STAT3 inhibitors are in clinical trials, such as TTI-101 (Tvardi Therapeutics) for treating advanced cancers, Napabucasin (BBI608) for gastric and pancreatic cancers, WP1220 (Moleculin

Biotech Inc.) and AZD9150 for lymphoma and other indications [311]. Given the involvement of STAT3' in inflammation and immune responses, it is plausible that these inhibitors could be explored for treating chronic inflammatory or autoimmune diseases in the future, although no specific clinical trials for such conditions have been reported to date.

EMH, which is the production of blood cells outside the BM, is frequently induced under stress conditions. EMH is generally anticipated as a compensatory mechanism that sustains hematopoiesis when the BM output is insufficient or serves to enhance overall hematopoietic capacity [171]. While EMH is most commonly observed in the spleen and liver—organs that serve as primary sites due to their role in embryonic hematopoiesis—EMH has also been detected in the lungs, brain, spinal cord, adrenal glands, and even in wounds [194, 312], highlighting its complex and not fully understood role. In our murine model of chronic inflammation, we observed that EMH occurs in spleen, but also at the sites of inflammation, such as paws and tails. Intriguingly, whereas EMH in cancer is typically associated with a myeloid-biased output [166, 313, 314], our transplantation assays of HSPCs isolated from both the spleen and paws of CMO mice revealed a significant bias toward lymphoid lineage reconstitution. Remarkably, transcriptomic analysis showed that these EMH HSCs differ from their BM counterparts, notably exhibiting enhanced antigen presentation in a subpopulation marked by CD53 expression. We propose that this antigen-presenting capability facilitates the interaction with naïve T cells and Tregs, creating a pro-survival environment that helps preserving the HSC pool. This hypothesis is supported by our co-culture experiments and by Treg depletion studies, which resulted in reduced HSC functionality. Overall, our observations allow us to propose a model in which EMH is not only compensating for inefficient BM hematopoiesis, but also contributing to modulate the excess of inflammatory signals.

Since HSCs are not typically associated with antigen presentation, the discovery of MHCII expression on these cells is a relatively novel phenomenon in the field [51, 53, 54]. It is incredibly fascinating that cells not typically associated with antigen presentation—such as fibroblasts, ECs, and HSCs [31]—can acquire these properties under certain stress conditions, including acute and chronic inflammation as well as cancer. Generally, MHCII expression on fibroblasts and ECs has been primarily studied in the context of cancer, where it is associated with immune evasion and the promotion of a suppressive tumor microenvironment through the

expansion of Tregs [30]. In HSCs, this acquired antigen-presenting capability may serve dual purposes: it may promote HSC's own survival and simultaneously dampen inflammation by inducing Tregs from naïve T cells or activating memory Tregs. As discussed in the "Non-professional APCs" section, current knowledge regarding MHCII expression on HSCs remains contradictory. Some studies suggest that MHCII on HSCs facilitates the presentation of neoantigens, leading to the elimination of aberrant HSCs while naïve T cells acquire a regulatory phenotype through interaction with HSCs [53]. In contrast, other studies propose that Tregs provide pro-survival signals to HSCs, thereby promoting the survival of certain HSC clones during aging [54]. However, it is unclear whether the function of MHCII is uniform across different conditions or whether its role varies with the inflammatory context. Moreover, since HSCs do not express classical co-stimulatory molecules such as CD80 and CD86, the molecular mechanisms by which they activate naïve T cells remain elusive—raising the possibility that alternative molecules may be involved. It is also uncertain whether HSC interactions with naïve T cells might promote the development of other T cell subsets. This area of research is particularly compelling and warrants further investigation. In the human context, the impact of MHCII expression on HSCs is similarly ambiguous. While MHCII-mediated antigen presentation might support the survival of EMH HSCs, blocking MHCII could potentially prevent the persistence of those cells that might contribute to disease pathology. Conversely, if these EMH HSCs exhibit superior functionality compared to BM HSCs, targeting them for therapeutic transplantation may offer clinical benefits. Notably, MHCII expression has been observed in leukemic patients, suggesting that it may promote leukemic cell survival and contribute to relapse [53]. These critical questions underscore the need for further research, and the antigen-presenting properties of HSCs must not be overlooked.

Another aspect worth discussing in this section is the distinct innate immune responses in CMO mice across three different genetic backgrounds (C57Bl/6J, C57Bl/6N, and BALB/c). Here, we addressed a significant gap in our understanding of how genetic variability influences sterile chronic inflammation. Although differences in adaptive immune responses among mouse strains (e.g., BALB/c being more Th2-prone and C57Bl/6J more Th1-biased) are well established [270-274], it remains largely unknown whether the genetic background similarly affects innate immune responses in autoinflammatory disorders, which are predominantly

driven by the innate immune system. Our findings indicate that both the development of osteomyelitis and the biology of neutrophils—the primary drivers of disease in CMO mice—vary significantly with genetic background. These observations emphasize the critical importance of carefully selecting mouse strains for specific studies. Although different strains may be used to investigate the same biological topic, our results demonstrate that outcomes are not directly interchangeable across strains due to inherent genetic variability. Consequently, it is essential to choose an appropriate mouse model tailored to the study's objectives, as the genetic background can significantly influence immune responses and disease progression. Finally, whether the genetic background also affects the development of EMH is a subject that also needs to be addressed in the future.

In conclusion, there are still significant gaps in our understanding of the impacts of chronic inflammation on hematopoiesis, BM and EMH HSCs, and on the immune system as a whole. We believe that the findings presented in these three publications shed important light on the understanding of the CMO pathology and provide a foundation for future research aimed at fully elucidating these complex processes and developing targeted therapeutic interventions to treat autoinflammatory diseases in patients.

5. CONTRIBUTION TO THE PUBLICATIONS

Publication used in this thesis:

Chronic inflammation decreases HSC fitness by activating the druggable Jak/Stat3 signaling pathway. Grusanovic S, Danek P, **Kuzmina M**, Adamcova MK, Burocziova M, Mikyskova R, Vanickova K, Kosanovic S, Pokorna J, Reinis M, Brdicka T, Alberich-Jorda M. EMBO Rep. 2023 Jan 9;24(1):e54729. doi: 10.15252/embr.202254729.

I joined this project at the start of my PhD. While the majority of the work was carried out by Srdjan Grusanovic, I contributed primarily through *in vivo* experiments and during the revision process. Specifically, I performed HSC transplantation assays and additional mouse analyses, collected samples for ELISA, conducted the ELISA assays, and carried out qPCR assessments of genes affected by the CMO BM niche. My contributions are depicted in the Figures.: EV3, 6, EV5 and EV7 of the manuscript.

HSCs and Tregs cooperate to preserve extramedullary hematopoiesis under chronic inflammation. Maria Kuzmina, Srdjan Grusanovic, Jiri Brezina, Mirko Milosevic, Karolina Vanickova, Nataliia Pavliuchenko, Sarka Ruzickova, Jakub Rohlena, Dominik Filipp, Katerina Rohlenova, Tomas Brdicka, Meritxell Alberich-Jorda. bioRxiv 2025.02.05.636492; doi: doi.org/10.1101/2025.02.05.636492

This project represents my main PhD work, during which I was responsible for generating ideas, designing experiments, performing the research, and analyzing the data. My contributions include phenotyping HSPCs in extramedullary sites, performing HSPC transplantation (from both spleen and paw), conducting partial single-cell RNA sequencing analysis, and executing co-culture experiments—from the isolation and preparation of HSPCs to the evaluation of the cultures. Additionally, I treated mice with an anti-CD25 antibody, assessed the treatment outcomes, and carried out subsequent transplantation experiments. I also wrote the manuscript with my supervisor, Meritxell Alberich-Jorda.

Genetic background affects neutrophil activity and determines the severity of autoinflammatory osteomyelitis in mice. Pavliuchenko N, **Kuzmina M**, Danek P, Spoutil F, Prochazka J, Skopcova T, Pokorna J, Sedlacek R, Alberich-Jorda M, Brdicka T. J Leukoc Biol. 2024 Dec 31;117(1):qiae168. doi: 10.1093/jleuko/qiae168. PMID: 39120532.

This publication was part of a GAUK project led by my colleague Nataliia Pavliuchenko from the Leukocyte Signaling Lab at IMG. I joined this work as a co-researcher within the framework of the GAUK project. I contributed to the realization of experiments, which included mice processing, flow cytometry measurements and setup, preparation and execution of migration assays, and preparation of the figures. My contribution is reflected in Figures 1 and 2 of the manuscript.

I hereby confirm that the author of the thesis, Maria Kuzmina, has substantially contributed to the publications listed above. Regarding her first-author manuscript, she performed the majority of the experimental work and contributed to the manuscript preparation.

……

Assoc. Prof. Meritxell Alberich-Jordà, Ph.D.

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7. REPRINTS OF PUBLICATIONS