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Interleukin-1α and Leukemia Inhibitory Factor Promote Cancer-Induced Extramedullary Hematopoiesis

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Dedicated to my love, Christine Horan

ABSTRACT OF THE DISSERTATION

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Professor Kyunghee Choi, Chairperson

Hematopoiesis, the formation of blood and immune cells from stem and progenitor cells, is a central biological process that alters in response to the organismal state. Many cytokines that accompany diseases have an impact on the rate, cellular output, and underlying mechanisms of hematopoiesis. Deepening our understanding of the alterations to hematopoiesis that occur with disease may offer therapeutics that normalize hematopoiesis and promote health. One component of hematopoiesis that is often overlooked are the non-hematopoietic cells of the stem cell niche that have crucial roles in affecting the parameters of hematopoiesis. While progress in understanding the hematopoietic stem cell niche during homeostasis has been made, how niche interactions change during pathologic conditions is less well understood. A central focus of my graduate studies has been to illuminate the interaction between pathological states, in particular solid tumors, and the hematopoietic niche.

In this dissertation, I have examined the relationship between hematopoietic stem and progenitor cells (HSPCs) and their splenic niche during solid tumor-induced extramedullary hematopoiesis (EMH). In this work, I identify that multiple models of murine solid tumors expand the splenic HSPC pool. Further studies of the PyMT-B6 transplantation model of breast cancer identified an inflammatory gene signature characterized by TNFα expression in extramedullary HSPCs in the presence of tumor, and I show a role for IL-1α derived from the tumor itself in producing this gene signature. To understand the impact of HSPC-produced TNFα, I developed a culture system for splenic stromal niche cells and demonstrate their activation in response to TNFα. Additionally, I demonstrate the expansion of the stromal EMH niche through tumor-derived Leukemia Inhibitory Factor (LIF) and its induction of proliferation within the splenic stromal niche. Using public data of human tissue RNA expression, with or without tumor, I show that multiple human cancer types share upregulation of both IL-1α and LIF. Finally, I show that the presence of both IL-1α and LIF has a combinatorial activation of EMH.

Together, the data presented in this dissertation centers the spleen as a hub of altered hematopoiesis during pathology and identifies two cytokine targets produced by solid tumor that modulate the state of the splenic niche to facilitate expansion of hematopoiesis to the spleen. In the future, this research may lay the groundwork for new avenues of niche-directed therapies and further exploration of EMH accompanying inflammatory pathologies.

Chapter 1: Introduction

1.1 Hematopoiesis

Hematopoiesis is the continuous process by which cells of the blood and immune systems are produced by the actions of hematopoietic stem cells. Thought to be organized similarly to an elevated pool of water draining into a branching system of rivers, hematopoietic stem cells maintain a pool of quiescent and activated stem cells that produce more differentiated and committed progenitor cells. These progenitors can continue to self-renew and differentiate until terminally differentiated cells are produced. The list of cellular or subcellular derived from hematopoiesis is long and growing, but famously includes red blood cells, platelets, T-cell lineages, B-cell lineages, monocyte lineages, and neutrophils. Working behind this system of hematopoietic stem cells are the actions of supporting cells known as the stem cell niche. The constitutive cells of the niche are drawn from both hematopoietic and non-hematopoietic lineages and have unique but sometimes overlapping roles. An understanding of the blood, immune, and hematopoietic system is central to understanding many clinically relevant pathologies.

1.1.1 History of the study of hematopoiesis

The origins of an understanding that a stem cell based system of hematopoiesis exists can be traced back to early studies on the shared blood types of co-placental fraternal twins and the potential for skin grafts between twins (*1*, *2*). Continued study of these mechanisms led to tolerance induction in mice by hematopoietic cell infusions and the 1960 Nobel Prize in Medicine (*3*). Efforts to understand and treat radiation syndrome in light of potential deaths from hematopoietic failure in civilians from Nagasaki and Hiroshima drove the development of hematopoietic cell

transplantation (*4*). This eventually drove the understanding that transplantation of bone marrow containing matching major histocompatibility complexes allowed the immune acceptance of skin grafts (*5*). Following the development of bone marrow chimeras was also the potentially the first report of graft-versus-host disease (*5*). Two scientists, Till and McCulloch, pioneered the modern study of the hematopoietic stem cells with a series of observations such as the clonal production of mixed hematopoietic progeny and selected self-renewal capacity (*6*). In the process of looking for the identity of hematopoietic stem cells by developing assays for different blood lineages, stromal cell co-cultures systems capable of supporting hematopoiesis were developed (*7, 8*). These co-culture systems develop contemporaneously with the first discussion of the hematopoietic stem cell niche (*9*). The expansion of monoclonal antibodies against leukocyte antigens helped define fully differentiated immune cells. When combined with assay systems for development of specific lineages, it was determined that precursors lacked some of the markers of their differentiated progeny, which in mouse cells would later be defined as the lineage panel (*10*). Conversely, in mice, the marker Sca-1 was able to segregate progenitor cells possessing all clonogenic potentials into the positive population (*11*). Defining mouse hematopoietic stem cells as Sca-1 positive was later followed by identification of c-KIT as a marker for hematopoietic stem cells (*12*). In humans, this was parallel by the identification of CD34+ marrows cell as capable of reconstituting the hematopoietic system of lethally irradiated baboons (*13*). Additionally, IL-7 receptor positive cells were found to produce all lymphoid fates while IL-7 receptor negative cells produced only myeloerythroid fates (*14, 15*). These historic scientific developments form the basis of study for my research and others across the field of hematopoiesis.

1.1.2 Evolution of mammalian hematopoiesis

Early hematopoietic systems began with hemocytoblasts in coelomic invertebrates that were capable of producing multiple cell types. In fact, several key mammalian hematopoietic transcription factors, *Gata*, *Fog*, and *Runx*, share orthologues with species as distantly related as *Drosophilia* and co-ordinate the production of phagocytic plasmatocytes, immunometabolic granulocytes, and red blood cells (*16*). Invertebrates phylogenetically related to vertebrates, the cephalochordates, potentially generate their hematopoietic cells in an aorta-gonad-mesonephros-like region but lack a closed circulatory system (*17*). The evolution of the hematopoietic system is thought to be closely linked to the development of a closed circulatory system and endothelial cell lineages at the evolutionary boundary between invertebrate and vertebrate species (*18*). In some invertebrate groups, a specialized blood cell, the amoebocyte, lines myoepithelial-based, open circulatory systems (*18*). This cell is hypothesized to have gained a more epithelial morphology consisting of the ability to secrete basement membrane proteins and intercellular junctions to begin the transition to a true endothelial cell (*18*). This evolutionary linkage between the hematopoietic and endothelial system forms an important developmental connection that is indicated by identification of the bipotent hematopoietic/endothelial hemangioblast (*19*). Tunicates, the most closely related invertebrate to vertebrates, have myeloid cells and graft-resisting cytotoxic cells, potentially similar to NK cells (*20*). Jawless chordates, in particular the lamprey, begin to strongly resemble mammalian immune system with their circulating myeloid, erythroid, and lymphoid lineages and their capability to produce antibodies, develop immunologic memory, and reject allografts (*21*). Furthermore, the lymphoid compartment of lampreys has three antigen receptors that are individually expressed on cells resembling mammalian B-cells, αβ-T-, and γδ-T-cells (*22*). Cartilaginous fish, including sharks, have the first phylogenetic instance of a spleen which also

functions as the major hematopoietic organ of the adult animal (*23, 24*). In bony fishes, as modeled by the zebrafish, hematopoiesis begins to undergo developmental waves (*21*). These steps of added complexity to the process of hematopoiesis mirror the evolutionary development of the animals they are found within.

1.1.3 Development of hematopoiesis across mammalian life stages

Mouse hematopoiesis reaches its mature adult state through three developmental waves. In the first wave, hematopoietic cells develop initially in the blood islands of the yolk sac around embryonic day 6.5-7 and have erythoid, macrophage, and megakaryocyte potential (*25-28*). Only erythroid potential is present in the yolk sac on embryonic days 7.5-8.5 (*26*). On embryonic day 8.5, the second wave of hematopoiesis begins with endothelial-to-hematopoietic transformation of yolk sac vascular beds into erythromyeloid progenitors (*29-33*). This wave gives rise to tissue-resident macrophage progeny that are maintained by self-renewal throughout life (*34-38*). The final wave is the definitive wave of hematopoiesis beginning on embryonic day 9.5 from endothelial-to-hematopoietic transition of cells near the dorsal aorta and contains the first multipotent progenitors capable of producing lymphocytes (*39-44*). Concurrent with the development of the definitive hematopoietic stem cell line is the formation of a recognizable stromal niche in aorta-gonad-mesonephros (*45*). Some lineages of lymphoid cells are only produced during embryonic development (*46-48*). Following development of hematopoietic stem cells, they then migrate to the fetal liver and continue to proliferate and mature (*49*). Stromal cells of the fetal liver play an important role in maintaining hematopoietic stem cells in this environment (*45*). The fetal spleen begins to support hematopoietic progenitors as early as embryonic day 12.5 with definitive hematopoietic stem cells arriving on embryonic day 15.5 following the decline of the fetal liver (*50, 51*). The bone marrow is seeded by hematopoietic stem cells beginning around

embryonic day 17.5 (*52*). Splenic hematopoiesis continues under homeostasis until 2 weeks after birth when only B-cell and erythrocyte development remains (*53, 54*). An important lesson from understanding developmental hematopoiesis is that the type of cells produced by and the location of hematopoiesis can undergo rapid and dramatic shifts and is accompanied by changes in the surrounding niche environment.

1.1.4 Hematopoietic stem cell dynamics

My preferred model of hematopoietic stem cell differentiation is a continuous, hierarchical model whereby quiescent stem cells can activate before potentially undergoing incremental steps of differentiation with the limited self-renewal present along the way (*55, 56*). This model of hematopoiesis has emerged coincidentally with studies using novel single cell RNA-sequencing technology where, contrary to previous models and techniques, obvious cell type boundaries have not been identified (*57*). At the top of this hierarchy is the quiescent hematopoietic stem cell. Quiescence is the process by which cells can remove themselves from the cell cycle (*58*). For hematopoietic stem cells, this pool of quiescent cells protects the hematopoietic system from genotoxic insults, viral infections, and progressive transformation by long-term replication while providing a safe haven of cells to re-establish hematopoiesis if required (*59*). These quiescent cells can identified within the pool of hematopoietic progenitors by the presence or absence of surface markers. The marker EPCR is a common positive selection marker for the quiescent hematopoietic stem cell population (*60, 61*). Many internal regulators are important for the maintenance of quiescence (*59*). One recently identified factor with profound effects in controlling quiescence is Hepatic Leukemia Factor (*62*). The process of transitioning between states of activation and quiescence is a highly regulated process influenced by internal and external factors acting on the cells. In particular, the hematopoietic niche has been shown to influence the activation state of

hematopoietic stem cells. One niche factor involved in enforcing quiescence is CXCL12 (*63*). One factor that has been identified to enforce activation during recovery from chemical injury to hematopoiesis is TGFβ (*64*). Upon activation, hematopoietic stem cells change their surface phenotype. Previous work has shown that CD34 is an activation marker for hematopoietic stem and progenitor cells. Indeed, research shows that murine CD34- cells becomes CD34+ upon cytokine activation or culture, CD34+ cells have long-term engraftment potential, and CD34+ cells become CD34- once engraftment has completed (*65, 66*). Additionally, expression of CD48 is recognized as a marker of activated stem cells (*67, 68*). However, discussion of surface markers must be accompanied by an understanding that these states are often reactive to environmental changes (*69*). Understanding the dynamics of the hematopoietic stem cell provides a foundation for understanding how it can differ between states of homeostasis and pathology.

1.1.5 Myelopoiesis in homeostasis and disease

The production of myeloid lineage cells, predominantly neutrophils and monocytes, from hematopoietic stem cells is a process that occurs during homeostasis but is also highly reactive to the organismal state (*70, 71*). The development of myeloid cells is a complex balance of select transcription factors throughout differentiation space (*72*). The list of transcription factors favoring myeloid differentiation include PU.1, CCAAT/enhancer binding proteins (C/EBPα, C/EBPβ and C/EBPε), growth-factor independent 1 (GFI1), and interferon-regulatory factor 8 (IRF8) (*73-78*). At the intersection of the hematopoietic stem cell and myeloid differentiation is PU.1 followed by IRF8 delineating monocyte lineage and C/EBPα delineating neutrophil lineage (*73, 74, 78*). In times of organismal stress, the nature of factors driving myelopoietic differentiation can be altered. For instance, mice lacking *Cebpb* maintain normal granulocytopoiesis at homeostasis but fail to engage emergency granulocytopoiesis during challenge (*79*). Cytokine factors can stimulate the

increased production of myeloid cells and include G-CSF, GM-CSF, M-CSF, IL-1, and IL-27 (*80-84*). Accompanying the ability of myelopoiesis to be expanded, changes to the cytokine milieu also impact the cellular products of myelopoieis. Most prominently is the production of an ill-defined class of myeloid cells associated with immunosuppressive activity, the myeloid-derived suppressor cell (*85*). These cells have attained a central role in modulating the immune microenvironment of solid tumors through their promotion of angiogenesis, metastasis, and reduced responsiveness to immune checkpoint blockade (*86-88*). One major question regarding myeloid-derived suppressor cells is how they develop. Current thought implicates many factors required for stimulating emergency granulocytopoiesis along with a second factor to direct their activation state towards an immunoregulatory phenotype (*89*). Two particularly important activation signaling pathways are NF-κB and STAT1. Factors implicated in inducing immunosuppressive activity through NF-κB are TNFα, IL-1β, and Toll-like receptor ligands while IFNγ is most commonly linked to STAT1 activation (*90, 91*). With a central role in both preventing and promoting disease, myelopoiesis is an important process with potential therapeutic implications.

1.1.6 Mobilization of hematopoietic stem and precursor cells

At homeostasis, hematopoietic stem and precursor cells are mobilized into the peripheral blood in small number and following the action of certain factors, this mobilization is able to be increased dramatically (*92*). Clinically, the ability to mobilize stem cells has been a boon for harvesting stem cells for transplantation (*93*). The most prominent inducers of stem cell mobilization are G-CSF, known clinically as filgrastim, and a CXCR4 inhibitor, plerixafor. In addition to its myelopoietic effects, G-CSF is thought to mobilize stem cells from the bone marrow by attenuating adhesions to the niche through protease dependent and independent mechanisms (*94-99*). Plerixafor, which

inhibits CXCL12-mediated retention and quiescence, was shown to increase stem cell mobilization by 50% in combination with G-CSF when compared to G-CSF alone (*100*). Other clinically utilized stem cell mobilizers include GM-CSF and KIT ligand (*93*). Additionally, there exist several more experimental mobilization agents. CXCR2 ligands, including CXCL1 and CXCL2, have long been recognized for their mobilization capability (*101*). Combining anti-VLA4 agents with CXCR2 ligands has shown to increase efficacy in mobilizing hematopoietic stem cells (*102*). What is of particular interest regarding mobilizing hematopoietic stem cells is the ways in which counteracting the actions of the bone marrow niche are important for this process.

1.1.7 Bone marrow hematopoietic niche

The bone marrow hematopoietic stem cell niche is a complex microanatomical environment that fosters the differentiation and self-renewal of hematopoietic stem cells while also directing their response to organismal changes (*103, 104*). While many cells have been implicated as possessing niche function, here we will discuss perivascular stromal cells, endothelial cells, sympathetic nerves, and macrophages. Pre-eminent within the bone marrow niche are a cell type known as perivascular stromal cells, mesenchymal stem cells, or osteoblast precursor cells. Often treated as multiple distinct cell types, here they will be treated singularly as modern techniques do not support their separation. What makes these cells stand above other constituents of the bone marrow niche is their expression of both CXCL12 and KIT ligand (*63, 105*). These mesenchymal stem cells in the bone marrow are most commonly marked by leptin receptor, nestin, Mx1, Prx1, Osx, PDGFRα, or CD51 (*106-108*). Initial studies also identified osteoblasts as critical components of the niche (*109, 110*). However, when CXCL12 or KIT ligand was deleted from mature osteoblasts, no significant changes to hematopoietic lineages were observed (*105, 108, 111*). This likely reflects the fact that mesenchymal stem cells of the bone are well recognized as being capable of

differentiating into osteoblasts *in vitro* and have been misconstrued as representing mature osteoblasts in early studies (*112*). In fact under the proper culture conditions, mesenchymal stem cells are capable of maintaining hematopoietic stem cells *in vitro (106, 107)*. Endothelial cells of the bone marrow contribute multiple niche factors including E-selectin, basic FGF, DLL1, IGFBP2, angiopoietin 1, DHH, and EGF (*113-118*). Within the bone marrow, the vascular niche is thought to be split into an arteriolar and a sinusoidal-megakaryocyte component. The arteriolar niche was first identified as the preferential localization of quiescent hematopoietic stem cells in the endosteal region of the bone marrow (*119*). In addition to the endothelial cells and mesenchymal stem cells, the arteriolar niche include sympathetic neurons and nonmyelinating Schwann cells, each with their own niche contribution. Sympathetic neurons alter CXCL12, angiopoietin 1, KIT ligand, and VCAM-1 expression of mesenchymal stem cells through β3-adrenergic receptor signaling and thus enhance mobilization (*120, 121*). Schwann cells contribute activated TGFβ, a regulator of hematopoietic stem cell quiescence (*122, 123*). The venous sinusoidal niche, in addition to the endothelial and mesenchymal stem cell components, is also defined by the presence of megakaryocytes that dampen hematopoietic stem cell proliferation through CXCL4 and TGFβ but also promote recovery after radioablation through FGF1 (*124-127*). Macrophages also have a niche function through their regulation of CXCL12 expression on mesenchymal stem cells (*128, 129*). The bone marrow niche also responds to a variety of signals including circadian rhythms, prostaglandins, pathogen-associated molecular patterns, and hormones (*120, 130-133*). A cruel irony of the bone marrow niche is that in a model of primary myelofibrosis, overgrowth of mesenchymal stem cells closes the marrow space for hematopoietic cells (*134*). When taken together the components of the bone marrow niche supply many factors,

often in conflict with each other, that react to the organismal state to maintain continued hematopoietic function.

1.1.8 Extramedullary hematopoiesis

For this document, extramedullary hematopoiesis is the expansion of hematopoietic potential to an organ space outside of the bone marrow in a mature animal and in response to altered organismal state. For instance, this definition excludes discussion of hematopoiesis occurring in organs like the thymus at homeostasis and in any organ during development. Three general circumstances surround the development of extramedullary hematopoiesis: (1) trapping of proliferative hematopoietic progenitors in the spleen during hyposplenism; (2) impairment hematopoietic capacity in the bone marrow due to damage or myelophthisis; or (3) abnormal levels of circulating factors with extramedullary hematopoietic capabilities (*135*). The most common organs for extramedullary hematopoiesis are the spleen, liver, and lymph nodes, but organs as diverse as the skin, the pleura, the adrenal gland, and the pancreas have reports of internal extramedullary hematopoiesis (*136-138*). Clinically, the majority of non-hepatosplenic instances of extramedullary hematopoiesis present with symptoms (*139*). For factor-induced extramedullary hematopoiesis, hematopoiesis active cytokines and pathogen-associated molecular patterns including G-CSF, GM-CSF, IL-3, and IL-6-sIL-6R complexes, and lipopolysaccharide and Pam3CSK4, respectively, have identified roles in stimulating extramedullary hematopoiesis (*140-143*). Some instances of extramedullary hematopoiesis induced by these factors are remarkable. Human IL-3 injected cutaneously into cynomolgus monkeys was reported as inducing cutaneous hematopoiesis with trilineage potential at the injection site (*143*). A patient was reported as having trilineage cutaneous hematopoiesis following G-CSF therapy to treat myelofibrosis (*144*). However, one should be cautious in avoiding the assumption that extramedullary hematopoiesis

copies all aspects of bone marrow hematopoiesis. Extramedullary hematopoiesis seems to occur in humans as a result of a loss of hematopoietic capacity in the bone marrow. This comports with the fact that extramedullary hematopoiesis is most commonly secondary to hematologic malignancy (*138*). When discounting hepatosplenic hematopoiesis, the most common condition was myelofibrosis with myeloid metaplasia and the most common location was the thoracic vertebral column (*139*). Most often extramedullary hematopoiesis is associated with myeloid- or erythroid-biased differentiation. However, transgenic models have reported extrathymic T-cell development (*145*). Mobilization of hematopoietic stem cells often coincides with extramedullary hematopoiesis and is likely important in seeding cells for the process. Whether proliferation of local progenitors contributes to extramedullary hematopoiesis is an open question. All things considered, extramedullary hematopoiesis is a fascinating phenomenon that is relevant to clinical medicine and offers numerous opportunities to further our understanding of hematopoiesis itself.

1.1.9 Extramedullary hematopoietic niches

Just as the bone marrow requires a niche to support hematopoietic stem cells, so too do extramedullary sites of hematopoiesis. Niche components representing the diversity of all sites of hematopoiesis are very poorly characterized. However, some aspects of the splenic hematopoietic niche in adult animals have been studied with even fewer studies of the liver and skin niche. Central to splenic hematopoiesis are still thought to be the mesenchymal stem cell and the endothelial cells. In the spleen, mesenchymal stem cells and endothelial cells both produce KIT ligand while only mesenchymal stem cells produce CXCL12 (*146*). Additionally, extramedullary hematopoiesis of the spleen has been localized to near sinusoids of the red pulp (*146*). In a liver model of extramedullary hematopoiesis, sinusoidal endothelium were observed upregulating CXCL12 (*147*). A case report of an adult patient with nodular, cutaneous extramedullary

hematopoiesis had KIT ligand and CXCL12 double positive cells within the lesion (*148*). Despite these similarities, a couple of tissue-specific differences are known about splenic mesenchymal stem cells including they are leptin receptor negative and express Tlx1 (*149*). Additionally, some supporting cell types of the spleen appear to be different. For instance, decreasing NK cells in the spleen increased myeloid progenitors, suggesting that NK cells negatively regulate splenic hematopoiesis. Whereas T-cells in the spleen can act as hematopoietic niche cells (*150, 151*). Finally, macrophages have a role in supporting erythropoiesis and hematopoiesis in the spleen (*152-155*). Together, what little is known about extramedullary hematopoiesis suggests that certain core factors are required for hematopoiesis in any organ, but that there exists organ specific factors that can modulate these core processes.

1.2 Organs of the hematopoietic system

The organs of the hematopoietic system vary dramatically in their structure and function yet are linked by their connection to generating and housing hematopoietic stem and precursor cells. Here, I review the two organs most relevant to my work though a complete treatment of this topic would also include discussions of the liver, lymph nodes, thymus, and peripheral blood.

1.2.1 Bone

There are about 206 bones in an adult human following fusions of the 270 bones present at birth, and each bone of the human body that is not a mirror image of another bone has unique morphology (*156*). In simple terms, the tissue architecture of the bone can be split into a biomechanical, load bearing segment with reduced cellularity and an internal soft tissue segment with high cellularity (*157*). For different bones and different life stages, the relative amount of these two segments may vary as bone remodeling is a continuous process (*158*). In addition to

their roles in structural support and hematopoiesis, bones also aid in maintaining acid-base and mineral homeostasis (*159*). Mineralized bone has two separate structures that represent their differing functions: cortical bone and trabecular bone. Cortical bone is dense, nearly continuous, and riddled with cylindrical units of laminated bone. This section is load bearing and has differing properties depending on whether the surface faces the marrow space or the surrounding tissue. The external, periosteal, surface is important for outward growth and fracture repair due to bone formation outweighing resorption while the internal, endosteal, surface is larger and expands the marrow space as bone resorption outweighs bone deposition. Trabecular bone is positioned within the marrow space and formed from a meshwork of bone. The bone marrow is located around the trabecular bone. Bone homeostasis is maintained by the opposing actions of two cell types: osteoclasts, a specialized macrophage, that resorbs bone; and osteoblasts, a differentiated stromal cell, that deposits collagen and minerals to form bone (*158*). Bones have a complicated network of vascularization that matches the morphology and function of the individual bone. 10 to 15% of resting cardiac output is directed to the bone (*160*). For smaller and flatter bones, nutrients and oxygen may be supplied by arteries on the periosteal surface, but larger bones may have multiple arties that pierce the cortex to provide vascular support to different areas of the bone. Smaller caliber vessel of the marrow cavity are dense branching networks of fenestrated sinusoids (*160*). Oxygen tension in the bone marrow varies based on the distance from arteriolar vessels with a mean pO2 of 6.6%, comparable to 3 to 9% pO2 in healthy tissues, to a mathematically predicted value of 1% in distal capillary beds (*161, 162*). Together, the anatomy of bone is a heterogenous structure filled with hard and soft tissue that balances the needs of highly metabolically active cell types with competing functions.

1.2.2 Spleen

The spleen is a peritoneal organ located on the left side between the diaphragm and upper stomach that serves a plethora of functions, primarily hematologic. The spleen begins development in the mouse on embryonic day 10.5-11 as a condensation of mesenchyme underlying the splanchnic mesodermal plate that directs spleno-pancreatic development (*163-167*). Several splenic developmental transcription factor are known including *Bapx1*, *Nkx2-5*, *Tlx1*, *Pod1*, *Wt1*, and *Pbx1 (164, 167-170)*. Though not considered a vital organ, failure to a develop a spleen is known as asplenia and increases the risk of sepsis 10- to 20-fold which can be life threatening in children (*166*). Structurally, the adult spleen is given its shape by a fibrous capsule with internal trabeculae (*166*). Internally, the spleen is split broadly into a white and red pulp with further functionally distinct regions. The organ is vascularized by the splenic artery that enters the spleen before branching into progressively smaller vessels before terminal arterioles release their contents into the open vasculature of the red pulp. The white pulp are lymph node like accumulations of white blood cells that ensheath the central arteriole before it release blood into marginal zone separating the white and red pulp. Though these white pulp structures have defined T-cell and B-cell zones like a lymph node, the fluid surveyed by these structures comes only from the blood. To enter back into circulation, red blood cells and other cell types must travel back into venous sinusoids. Red blood cells that cannot pass through the walls of the sinusoids due to age, damage, infection, complement deposition, or antibody deposition are phagocytosed by specialized macrophages (*171*). Speaking generally, the immune function of the red and white pulp match the functions of innate and adaptive immunity, respectively. During systemic infections, macrophages of the red pulp can phagocytose circulating pathogens and present the resulting antigens to the adaptive immune cells of the white

pulp (*166*). Additionally, the red pulp is considered to be the site of extramedullary hematopoiesis and a reserve for blood and immune cells to be recruited during emergencies (*171, 172*). One important physiologic feature of the spleen is slowness of the blood flow that allows for the immunologic and hematologic actions of the organ (*166*). All told, the spleen is a highly dynamic organ whose role in immunologic and hematologic processes adapt to meet the needs of the organism.

1.3 Cytokines

Instances of cytokines that possess hematopoietic activity are many and ever growing. For this review, I will focus on those that play a central role in understanding my data presented in the next chapter.

1.3.1 IL-1α and the IL-1 family

The IL-1 cytokine family has 11 members corresponding to 10 different receptors. Within this cytokine family are three subfamilies: IL-1, IL-18, and IL-36. The unifying features of cytokines in this family are their lack of a signal peptide for secretion, cytoplasmic distribution as precursor molecules, and the aliphatic amino acid – any amino acid – aspartic acid tripeptide consensus amino acid sequence nine amino acids from the optimal N-terminus for beta-barrel formation after cleavage and receptor binding (*173, 174*). Only the IL-1 receptor antagonist differs from other family members regarding these shared features as it lacks all of them. IL-1 family receptors signal through a trimeric complex of the specific IL-1 receptor, the IL-1 family cytokine, and a co-receptor. Binding of the cytokine with the primary receptor recruits the co-receptor and signaling can occur on the cytoplasmic side. For receptors with Toll-IL-1 receptor domains, this activates MyD88 and downstream NF-κB signaling. Additionally, the co-receptor

can present a soluble form, either cleaved from the cell surface or produced by the liver. IL-1α is considered a classic damage associated molecular pattern whose sensation can begin immune responses and its biology reflects that (*175, 176*). IL-1α signals through the IL-1R1 receptor and the co-receptor IL-1R3, both of which have Toll-IL-1 receptor domains. IL-1α is an unusual member of the IL-1 family as it is constitutively present in epithelial and mesenchymal cell types and does not require proteolysis to signal (*173*). However, IL-1α has a nuclear localization signal, and when apoptosis occurs, IL-1α traffics to the nucleus, bind chromatin and becomes immunologically silent. In contrast, when necrosis happens, IL-1α traffics to the cytoplasm where it will be immunologically active after the cell dies (*177*). With its unique biologic activity, it is no surprise that IL-1α has been reported to contribute to auto-immune disease, microbial infections, and cancer (*176*).

1.3.2 Tumor necrosis factor alpha

Tumor necrosis factor alpha (TNFα) is a quintessential cytotine with wide-ranging and pleiotropic functions and effects (*178*). TNFα is a trimeric member of the TNF family of cytokines. TNFα can signal through two receptors, TNFR1 and TNFR2. Downstream of signaling of TNFα is complicated and involves a menagerie of signaling mediators. Outcomes of TNFα signaling are highly context dependent but can include NF-κB activation, MAPK activation, or cell death. In the context of cancer, TNFα has roles at nearly every stage including tumorigenesis, tumor growth, angiogenesis, and metastasis but also includes anti-oncogenic effects and usage as an anti-cancer treatment (*178*). At the intersection of cancer and hematopoiesis, TNFα has been implicated in inducing myelopoiesis in the context of cancer (*179*).

1.3.3 Leukemia inhibitory factor

Leukemia inhibitory factor (LIF) is an IL-6 family member that signals through gp130 and the LIF receptor (*180, 181*). Signaling through LIF receptor is also shared with other IL-6 family members including OSM, CTNF, CT-1, and CLC, though all of these cytokines have additional receptors or co-receptors for signaling (*180, 182*). Signaling downstream of LIFR:gp130 is thought to be most prominent through JAK1, though JAK2 and TYK2 can bind as well (*183-186*). JAK1 activation lead to activation of STAT3, MAP kinase pathways, and PI3 kinase in amounts that appear to be cell type specific (*187-189*). The activity for which LIF is best known is maintaining mouse embryonic stem cells *in vitro (180).* Of these pathways, STAT3 and PI3 kinase both lead to enhanced self-renewal and inhibition of differentiation in mouse embryonic stem cells while MAP kinase activity activates differentiation (*190-193*). LIF has a number of interesting functions outside of embryonic stem cells. Mutations in LIF have been reported in infertile women that concurs with failure of LIF knock out dams to successful implant blastocysts and the high expression of LIF in endometrial glands (*194-196*). In neuronal and stromal cell types, LIF also seems to increase growth while altering differentiation. Overexpression of LIF by an injected cell line led to bone marrow fibrosis and elevated osteoblast numbers (*197*). Similarly, osteoblasts express LIF receptor and LIF enhances osteoblast differentiation while inhibiting adipocyte differentiation (*198*). LIF enhances neural stem cell self-renewal when overexpressed by adenovirus, induces astrocyte differentiation in culture, and stimulated proliferation of oligodendrocyte precursor cells when overexpressed by adenovirus (*199-201*). Myoblasts stimulated by LIF *in vitro* had increased proliferation but were inhibited in differentiating into myotubes while LIF enhanced recovery from muscle injury *in vivo (202, 203).* In cancer, LIF expression has been reported in many solid tumors such as colorectal, nasopharyngeal, skin, and

breast as well as supporting a variety of tumor functions (*180*, *204*). Even though exploration of the role of LIF is ongoing, several lines of evidence suggest that it is a potent cytokines with impact on proliferation and differentiation of stromal cells across the body.

1.3.4 KIT ligand

KIT ligand is a fundamental hematopoietic growth factor and ligand of the receptor c-KIT (*205*). KIT ligand has a soluble and transmembrane form (*206*). Mice that lack function in both forms have severe and fatal anemia due to hematopoietic failure along with pigment and germ cell deficiencies (*207*). Mice lacking only the transmembrane form are still anemic, lack pigmentation in the coat, and are sterile (*206*). Furthermore, the low body weight, anemia, and bone marrow cellularity in mice lacking transmembrane KIT ligand cannot be rescued by overexpression of soluble KIT ligand but can be with membrane-restricted KIT ligand (*208*). Conversely, enforcing membrane-restricted KIT ligand could not rescue reduced bone marrow myeloid progenitors in mice lacking transmembrane KIT ligand but overexpression of soluble KIT could (*208*). Total peripheral blood leukocytes were rescued by both (*208*). Finally, compared to wild-type mice, mice expressing KIT ligand missing the main cleavage site, and therefore lacking the majority of soluble KIT ligand, did not have differences in the number of hematopoietic progenitors in the bone marrow or in mature cells in the blood (*209*). Binding of KIT ligand to c-KIT dimerizes the receptor and activates its intrinsic tyrosine kinase activity (*210-212*). The different forms of KIT ligand also signal differently. Soluble KIT ligand signals rapidly and transiently followed by receptor degradation while the membrane-associated form has sustained signaling (*212*). Downstream this difference leads to membrane-associated KIT ligand inducing longer lasting ERK1/2 and MAP kinase activity (*213*). Potentially the inability of c-KIT to be internalized when bound to membrane-associated KIT ligand sustains signaling as agonism by immobilized anti-c-

KIT antibodies produce similarly sustained signaling (*214*). These data indicate that both forms of KIT ligand forms are functionally important but also are unique and that the transmembrane form may be the more important of the two. Despite the importance of c-KIT/KIT ligand for maintaining hematopoiesis, c-KIT signaling is also implicated in supporting quiescence of hematopoietic stem cells (*215*). Additionally, c-KIT is recognized as enhancing the signaling of the EPO receptor and IL-7 receptor, two important hematopoietic cytokines, as well as PDGFRα (*216-218*). KIT ligand and its receptor c-KIT clearly play a crucial role in hematopoiesis and have biology befitting their centrality.

1.3.5 Granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF) is a cytokine with potent activity on hematopoiesis (*219*). G-CSF signals through a private homodimeric receptor G-CSFR (*220*) . G-CSF levels are low during homeostasis but can rise during an immune response and then fall back to baseline (*221, 222*). Mice lacking either G-CSF or its receptor have severe neutropenia but do still produce a small amounts of neutrophils (*80, 223*). G-CSF is also able to mobilize hematopoietic stem cells into circulation and establish extramedullary hematopoiesis (*144, 224*).

1.4 Cancer

Cancer is a major health concern in the United States of America and globally. In the U.S.A., cancer is the second leading cause of death and costs more to treat than any other disease (*225*). Yet, this broad definition of cancer belies the deep complexities lying beneath the moniker. Any tissue type can undergo the highly individualized process of transformatiо into cancer. It is because of this vastness that cancer remains one of the most scientifically studied disease and has

been for many years. Here I but dip toes into the ocean of research surrounding cancer to illuminate the broad and the very narrow topics important to my work.

1.4.1 Hallmarks of Cancer

At the heart of cancer is the activation of continued proliferation in spite of normal checks to this process. Foremost to this process is sustained growth signaling. This may be due to autocrine signaling, induced paracrine signaling, or constitutive activation of internal signaling mechanisms. Conversely, cancers must also suppress pathways that would inhibit proliferation such as apoptosis or quiescence. Driving these changes to proliferative and anti-proliferative pathways are genetic changes that can mutate proteins directly or express normally repressed genes. In pursuit of continued growth, cancers must manipulate itself and their environment to allow growth such as by recruiting vasculature to supply nutrients or changing nutrient pathways as well as gaining or utilizing motility and invasion pathways to seek out and cultivate new sites for growth. Finally, cancers must manipulate the local and systemic immune environment to avoid recognition of its transformation and to promote immunologic states that enhance growth (*226*). Together these features simplifies and categorizes the intricacies governing cancer initiation and progression.

1.4.2 Immune environment of cancer

Cancer and the immune system exist in a dynamic tension with competing interests where tipping towards either extreme can be detrimental to organismal health. Still, manipulation of the immune system toward anti-cancer immunity has been the most exciting development in modern cancer therapy. Central to a modern understanding of anti-cancer immune activation is that mutations to the cancer genome can produce novel antigens recognized by the adaptive immune

system and that reduced expression of immune surveillance genes lead to NK cell activation (*227, 228*). Pro-cancer immune suppression is mediated by intrinsic mechanisms preventing chronic immune activation and cancer-induced immune cell phenotypic skewing away from activation (*227*). These competing signals set a balance that is now being manipulate by therapeutics such as immune checkpoint inhibitor, recombinant cytokine therapy, and chimeric antigen receptor T- and NK-cell therapies (*227, 229-231*). However, care must be taken as over-activation of the immune system from these therapies can lead to severe side effects (*232, 233*). Therapeutics developed with an understanding of cancer-immune interactions are undergoing a revolution but progress still remains in terms of efficacy and side-effects.

1.4.3 MMTV-PyMT model of breast cancer

Breast cancer is the most common cancer in people who are biologically female and leads to the second highest number of deaths from cancer (*234*). Mouse models of breast cancer have been developed to study this important disease process. The MMTV-PyMT mouse models of cancer is a genetic model where the mouse mammary tumor virus long terminal repeat promoter driving expression primarily in mammary epithelial cells is placed ahead of the polyomavirus middle T antigen oncogene leading to continuous growth signaling from this receptor tyrosine kinase analogue (*235*). Mice with this construct will develop hyperplasias followed by carcinoma *in situ* before progressing to carcinoma with a timeline dependent on the mouse strain and sex with female FVB/N being the most rapid (*236*). Tumors produced in this model have gene expression that resembles the human luminal B subtype (*237*). The MMTV-PyMT model has been used to understand how breast cancer and the immune system interact, in particular how the immune system restricts tumor growth while the tumor tries to shift the immune environment in its favor. For instance, RAG1 knockout mice, and thus mice lacking mature T- and B-cells, expressing

MMTV-PyMT have accelerated tumor onset (*238*). Additionally, IL-15 knockout mice with the MMTV-PyMT transgene experience accelerated tumor growth (*239*). On the other side of the coin, the MMTV-PyMT model has been shown to use G-CSF to foster development of immunosuppressive neutrophils at the expense of dendritic cells capable of cancer immunosurveillance (*240, 241*).

1.6 Summary

Hematopoiesis is a dynamic process in science history, in evolution, and in biology. The production of the cells from hematopoietic stem cells touch organismal physiology in nearly every regard, and to compete this herculean task, the hematopoietic stem cell relies on the continuous support of cells throughout the body, in particular its stromal companion – a friend since time immemorial. To us today, alterations and manipulations of hematopoiesis intertwine with the diseases that continue to scourge humanity despite the lifelong efforts of generations. In this document are described the effort of someone to grab hold of history and endeavor to lift the veil of ignorance and glimpse a tiny, blurry reflection of truth.

Chapter 2: Interleukin-1α and Leukemia Inhibitory Factor Promote Cancer-Induced Extramedullary Hematopoiesis

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2.1 Acknowledgements

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D.A.G.B. and K.C. conceived the study, analyzed the data, and wrote the paper. D.A.G.B. and J.W. performed the *in vivo* experiments. D.A.G.B. analyzed the scRNA-seq experiments. D.A.G.B., M.K., and A.K. conducted *in vitro* experiments. D.A.G.B. and M.S. analyzed human RNA-seq data. D.A.G.B. and K.K. created and purified lentivirus. D.A.G.B. and B.H. Z. imaged the spleen by confocal microscopy. C.L.S. provided the Lifr-flox mice.

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2.2 Abstract

The interaction between hematopoietic stem cells and their niche determines the outcome of hematopoiesis. While progress in understanding the hematopoietic stem cell niche during homeostasis has been made, how niche interactions change during pathologic conditions is less well understood. Here, we examined the relationship between hematopoietic stem and progenitor cells (HSPCs) and their splenic niche in tumor-induced extramedullary hematopoiesis (EMH). We identify an inflammatory gene signature characterized by TNFα in extramedullary HSPCs associated with cancer-induced EMH. We show a role for IL-1α in producing this gene signature and for HSPC-produced TNFα in activating EMH niche activity. We also demonstrate the expansion of the EMH niche through tumor-derived Leukemia Inhibitory Factor (LIF) inducing the proliferation of splenic stromal niche. Finally, we show that both IL-1α and LIF are upregulated in a subset of human cancers and have synergistic effects in activating EMH. Together, these data expand avenues for developing niche-directed therapies and further exploring EMH accompanying cancerous pathology.

2.3 Introduction

Hematopoiesis produces differentiated cell types of the blood and immune systems from hematopoietic stem and progenitor cells (HSPCs). Organismal changes such as disease can modulate the location and cellular output of hematopoiesis (*242, 243*). Expansion of hematopoiesis outside of the bone marrow (BM), known as extramedullary hematopoiesis (EMH), accompanies pathologic states and occurs mainly within the spleen and liver. Long underappreciated in human disease, EMH is now beginning to be recognized as important component to multiple hematologic and non-hematologic disease (*138, 244*). The induction of EMH requires mobilization of HSPCs from the BM by cytokines, such as ligands for CXCR2 including CXCL1 and CXCL2 (*101, 102, 245*). Clinically, EMH presents in a diverse set of solid tumors, including breast, lung, renal, colon, gastric, pancreatic, and prostate cancer (*246, 247*). Of particular interest is EMH in the spleen due to the organ's role in supplying myeloid cells during multiple injury and disease states (*172, 248*)

Myeloid-biased differentiation is a response of hematopoiesis to inflammatory signals, including IL-1β, TNFα, and G-CSF (*249-251*). Enhanced myelopoiesis, characteristic of EMH, can exacerbate diseases like solid tumors, arthritis, and myocardial infarction by increasing the number of cells that drive pathology (*247, 252, 253*). Clinically, increased myeloid cell production can be measured by an increased ratio of neutrophils (PMNs) to lymphocytes in the peripheral blood (PB). Across multiple tumor types, including breast, colon, pancreatic, and gastric cancer, as well as a systematic review of all cancer types, a high neutrophil-to-lymphocyte ratio is a poor prognostic factor for survival (*254-257*).

Like other stem cells, hematopoietic stem cells rely on supporting cell types known as the niche (*258, 259*). Essential to hematopoietic niche function is the production of membrane-bound KIT

ligand, a key growth factor for HSPCs (*260-262*). Additionally, the niche must produce factors to attract and adhere HSPCs. CXCL12 is a critical chemotactic factor for HSPCs within the BM niche while VCAM-1 is central to adherence through interactions with VLA-4 and other integrins on HSPCs (*63, 263*). Among hematopoietic niche cell types, perivascular stromal cells play a central role through their production of KIT ligand and CXCL12 (*105, 111*). Mesenchymal stem cells have been shown to exert niche function in both mice and humans (*106, 264*). Despite their importance to the niche, demarcating cells as perivascular stromal cells has been tricky. However, several schemas have recognized PDGFRα as an important marker and noted their co-expression of PDGFRβ (*107, 265, 266*). This PDGFRα+/β+ surface phenotype matches mesenchymal stem cells as identified by single-cell RNA-sequencing of limb muscles (*267, 268*).

Significant advances have been made in delineating the BM niche and HSPC interaction at homeostasis (*258, 259*). However, although perivascular stromal cells have been appreciated as contributing to the splenic niche at homeostasis (*146, 269*), HSPC niches outside of the BM that support EMH are less well-understood. Here we demonstrate the importance of splenic EMH in producing PMNs during a mouse model of breast cancer and identify a novel inflammatory phenotype for HSPCs conducting EMH. We delineate cytokine communication between IL-1α-inflamed, splenic HSPCs producing TNFα and their splenic niche. We also investigate a parallel mode of cytokine communication between tumor cells and the splenic niche through Leukemia Inhibitory Factor (LIF). Both pathways may increase the myelopoietic capacity of the spleen during cancerous pathologies.

2.4 Materials and Methods

2.4.1 Animals

Wild-type C57BL/6J mice (#000664), B6N.Cg-Tg(PDGFRa-cre/ERT)467Dbe/J (#018280), B6.SJL-Ptprca Pepcb/BoyJ (#002014), and B6.FVB-Tg(Cdh5-cre)7Mlia/J mice (#006137) were obtained from The Jackson Lab. MMTV-PyMT mice on a C57BL/6J background were a gift from Dr. M. Egeblad. *Lifr*-flox mice were obtained courtesy of Dr. Colin Steward (*270*). All mice used in experimentation were female between the ages of 8 and 16 weeks unless otherwise stated. Animal husbandry, handling, and experimentation were approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine.

2.4.2 Mouse tumor models

MMTV-PyMT transgenic mice were used as a spontaneous model of breast cancer and were analyzed when evidence of peripheral neutrophilia was present which was between 3 to 6 months. For tumor transplantation studies, $5x10^5$ PyMT-B6 tumor cells, $5x10^5$ LLC tumor cells, $2x10^6$ 1956, or $2.5x10^5$ PyMT-B6 gene knockout tumors cells were injected subcutaneously in a slurry of 1:1 EHS ECM growth factor-reduced gel (Corning, # 354230; Sigma, #E6909) to PBS into the flank of the mouse and harvested after 21 days for PyMT-B6, 16 days for LLC, 17 days for 1956, and 28 days for PyMT-B6 gene knockout experiments. PyMT-B6, wild-type and knockout, cells and LLC cells were grown in DMEM with penicillin/streptomycin, 10% fetal bovine serum, and 10mM HEPES buffer. 1956 cells were grown in RPMI-1640 with penicillin/streptomycin, 10% fetal bovine serum, 100mM sodium pyruvate, 7.5% v/v sodium bicarbonate, and 50μM beta-mercaptoethanol. Supernatants were collected after 24 hours of incubation in culture starting 2 days after passage.

2.4.3 Flow cytometry

Spleens were homogenized through a 100-μm filter. BM from femur and tibias was ejected by centrifugation at 3,200g for 2min at 4C. Peripheral blood was collected by cheek bleed. RBCs were lysed when needed using ACK lysis buffer (ThermoFisher, A10492-01). Cells were counted on an automated Nexcelom cell counter.

Cells were blocked with TruStain FcX PLUS anti-CD16/32 antibody (Biolegend, 156603) or anti-CD16/32 BV421 (Biolegend, clone 93) where appropriate before staining with antibodies followed by flow cytometry on a Gallios (Beckman Coulter) or a FACScan II (BD). When staining for intracellular cytokines, Cytofix/Cytoperm (BD, 554714) was used according to manufacturer's instruction and 1μg/mL brefeldin A was maintained in the FACS buffer until fixation. Viability staining was added according to manufacturer's instructions before beginning flow cytometry. Analysis was performed with FlowJo v10 software (Tree Star).

The following antibodies and reagents were purchased from BioLegend: anti-CD45.2 APC (clone 104), anti-CD11b APC-Cy7 (clone M1/70), anti-CD11b PE (clone M1/70), anti-Gr1 FITC (clone RB6-8C5), anti-Gr1 APC (clone RB6-8C5), anti-B220 PerCP/Cy5.5 (clone RA3-6B2), anti-B220 PerCP-Cy5.5 (clone RA3-6B2), anti-CD3e FITC (clone 145-2C11), anti-CD3e PE-Cy7 (clone 145-2C11), anti-Sca-1 APC (clone D7), anti-Sca-1 PerCP-Cy5.5 (clone D7), anti-CD45 AF700 (clone 30-F11), anti-CD45 BV421 (clone 30-F11), anti-c-Kit PE-Cy7 (clone 2B8), anti-c-Kit PE (clone 2B8), anti-VCAM-1 APC (clone 429), anti-PDGFRβ APC (clone APB5), anti-PDGFRα (clone APA5), 7-AAD dye (#420404), anti-IL-7R PE-Cy7 (clone A7R34), streptavidin PerCP-Cy5.5 (#405214), streptavidin BV421 (#405225), streptavidin APC (#405207), and biotin anti-lineage (#133307). Anti-CD34 FITC (clone RAM34) was purchased

from Thermo. Anti-CD45.1 PE (clone A20) was purchased from BD Biosciences. Anti-KITL biotin (#102501) and biotinylated goat IgG control (#105601) were purchased from R&D Systems.

Cell type delineations were made as follows: KSL cells were gated as $CD45^+/Lineage^-/c\text{-}Kit^+/Sca\text{-}1^+$; granulocyte-monocyte precursor (GMP) cells were gated as $CD45^+/Lineage^-/c\text{-}Kit^+/Sca\text{-}1^-/CD16/32^+/CD34^+$; common lymphoid progenitor (CLP) cells were gated as $CD45^+/Lineage^-/c\text{-}Kit^-/Sca\text{-}1^+/IL\text{-}7R^+$.

2.4.4 Colony forming assay

Peripheral blood or the full contents of ABS:hematopoietic progenitor co-culture 24-wells were plated into complete methylcellulose media (Stem Cell Technologies, M3434). Colonies were scored 7-14 days after plating.

2.4.5 Bone marrow transplant

For splenocyte transplantation, CD45.2 mice were irradiated with 9.5 Gy and $1x10^6$ splenocytes from CD45.1 control or CD45.1 tumor-bearing animals were injected intravenously by the retroorbital route 24 hours after irradiation. For niche function studies, CD45.2 mice were irradiated with 9.5 Gy and CD45.1 hematopoietic cells were isolated from cell culture with or without ABS cells and injected intravenously by the retroorbital route 24 hours after irradiation Mice were monitored daily for mortality or signs of severe morbidity up to 28 days. Mice were maintained until mortality to evaluate the long-term reconstitution potential.

2.4.6 Splenectomy

Splenectomies and sham surgeries were conducted courtesy of the Hope Center Animal Surgery Core, Washington University School of Medicine. After a week recovery period, mice were injected with PyMT-B6 tumor cells as detailed above.

2.4.7 Single cell RNA-sequencing and analysis

Spleens were minced and digested in 1mg/mL Collagenase Type IV + 0.25mg/mL DNase I. Bone marrow was removed by centrifugation as detailed above and digested. Digestion was quenched then filtered through a 100μm filter. Cells were pelleted, counted, and aliquoted. TruStain FcX™ PLUS was used to block samples then biotin anti-lineage antibodies were used to stain lineage cells. After washing, strepavidin magnetic beads (NEB, S1420S) were used to deplete lineage positive cells. Remaining cells were pelleted and then stained with streptavidin BV605 (Biolegend, #405229), anti-CD45 AF700, anti-PDGFRα APC, anti-CD51 PE (Biolegend, clone RMV-7), anti-CD31 PE-Cy7 (Biolegend, clone 390), anti-Sca-1 PerCP-Cy5.5 (Biolegend, clone D7), and anti-c-Kit FITC (Biolegend, clone 2B8). Cells were then washed into holding buffer (0.04% BSA in PBS), stained with DAPI, and sorted on a high modified MoFlo into five populations: Live/Lin-/CD45+/c-Kit+/Sca-1+, Live/Lin-/CD45+/c-Kit+/Sca-1-, Live/Lin-/CD45-/CD31+, Live/Lin-/CD45-/CD31-, Live/Lin-/CD45 /CD31-/CD51+, Live/Lin-/CD45-/CD31-/CD51-. These populations were combined at equal ratios and submitted for 10X Genomics 3' v3.1 Chemistry sample preparation and sequencing on a NovaSeq6000 at the Genome Technology Access Center.

Cell Ranger (10x Genomics, Pleasanton, CA) with default settings de-multiplexed, aligned, filtered, and counted barcodes and UMIs. SoupX preprocessing was used to remove ambient RNA contamination at a contamination fraction of 10% (*271*). Filtered outputs were imported into R

v4.0.5 using Seurat v3.2.3 and barcodes with fewer than 350 unique genes were excluded. Seurat objects from the four experiment groups were merged and an SCT transformation with a variable feature count of 20,000 was performed on the resulting object (*272, 273*). The dimensions of the object were reduced using RunPCA with principal coordinates equal to 50. UMAP coordinates were calculated using all 50 PCA dimensions and a minimum distance of 0.05. FindNeighbors function was used to compute nearest neighbors using all 50 PCA dimensions and FindClusters function at a resolution of 1.2 was used to compute cell clusters. Markers for each cluster were calculated using FindAllMarkers function with a minimum percentage of 0.1.

For reanalysis of a publically available single cell RNA-sequencing dataset of bone marrow niche cells (*274*), data was downloaded from GSE108891 on Gene Expression Omnibus. Raw counts files for GSM2915575, GSM2915576, GSM2915577, and GSM3330917 were imported into R using Seurat 3.2.3 and barcodes with fewer than 500 unique genes were excluded. Seurat objects from the four experiment groups were merged and an SCT transformation with a variable feature count of 8,000 was performed on the resulting object (*272, 273*). The dimensions of the object were reduced using RunPCA with principal coordinates equal to 20. UMAP coordinates were calculated using all 20 PCA dimensions and a minimum distance of 0.05. FindNeighbors function was used to compute nearest neighbors using all 20 PCA dimensions and FindClusters function at a resolution of 0.2 was used to compute cell clusters. Markers for each cluster were calculated using FindAllMarkers function on default settings.

2.4.8 Magnetic bead isolation and quantitative reverse transcriptase analysis

TruStain FcX™ PLUS antibody was used to block samples then biotin anti-lineage antibodes and biotin anti-Flk1 (Biolegend, clone 89B3A5) antibody were used to stain cells. After washing,

strepavidin magnetic beads were used to bind the stained cells. Positive cells were depleted by two rounds of magnetic selection. Depleted cells were pelleted and stained with anti-CD34 FITC and anti-FITC biotin (Biolegend, clone FIT-22). Cells were washed, pelleted, and resuspended before adding streptavidin magnetic beads. After incubation, the tubes were placed on the magnet and the supernatant removed. Using an RNeasy Kit Micro (Qiagen, #74004), RLT buffer was used to lyse the cells before proceeding with RNA isolation according to manufacturer's instructions. qScript™ cDNA SuperMix (QuantaBio, 95048-100) was used to produce cDNA before running RT-qPCR with 2x SYBR Green qPCR Master Mix (BiMake, B21203) according to manufacturer instructions. Primers sequences were as follows: Tnf forward - CCCTCACACTCAGATCATCTTCT, reverse - GCTACGACGTGGGCTACAG; Cxcl2 forward – CCAACCACCAGGCTACAGG, reverse – GCGTCACACTCAAGCTCTG; Nfkbia forward – TGAAGGACGAGGAGTACGAGC, reverse – TTCGTGGATGATTGCCAAGTG; Nfkbiz forward – GCTCCGACTCCTCCGATTTC, reverse – GAGTTCTTCACGCGAACACC; Mki67 forward – ATCATTGACCGCTCCTTTAGGT, reverse – GCTCGCCTTGATGGTTCCT; Il1r1 forward – GTGCTACTGGGGCTCATTTGT, reverse – GGAGTAAGAGGACACTTGCGAAT; Hprt forward – TCAGTCAACGGGGGACATAAA, reverse – GGGGCTGTACTGCTTAACCAG.

2.4.9 ELISA and multiplex protein assay

ELISA kits for IL-1α (Abcam, ab199076), CXCL1 (R&D, DY453-05), and CXCL12 (Abcam, ab100741) were used according to manufacturers' instructions. LIF serum samples were analyzed using the Abcam, ab238261, while all other sample types were analyzed using R&D, DY449. Serum samples from MMTV-PyMT mice and littermate controls were sent to Eve Technologies

(Calgary, AB, Canada) and assayed using the 44-plex Mouse Discovery assay. Results from Eve Technologies were imported into R, log10 normalized, and plotted using the heatmap.2 function in the gplots package.

2.4.10 In-vivo cytokine injection

TNFα (Peprotech, 315-01A) and IL-1α (Peprotech, 211-11A) was purchased, resuspended according to manufacturer's instructions. For TNFα and IL-1α experiment, 2μg and 0.5μg or 0.2μg per mouse were injected retroorbitally, respectively. Mice were analyzed 24 hours later.

2.4.11 CRISPR-Cas9 gene deletion in PyMT-B6

PyMT-B6 cells were seeded and then grown overnight to around 70% confluence before adding TrueCut™ Cas9 Protein v2 (Thermo, A36497), Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent, and TrueGuide™ Synthetic sgRNA (Thermo, #A35533) according to manufacturer's instructions. Guide RNAs from the manufacturers catalog were selected to be positioned in the earliest exon shared by all known isoforms and to minimize the distance between the two cut sites. Both guides were incubated with the cells during lipofection. After lipofection, cells with single cell cloned. Each clone was tested for deletion of the gene by ELISA, Sanger sequencing, and gel electrophoresis when applicable. *Csf3* was deleted initially then a successful clone was used as the parental line for subsequent deletion of *Lif* or *Il1a*. These knockout cell lines were injected in vivo as described above.

2.4.12 Splenic stromal cell isolation, culture, and co-culture with hematopoietic progenitors

Spleens were minced and plated on gelatin coated plates. Growth media for cells was alpha-MEM with 10% FBS, 1x Glutamax, 10mM HEPES buffer, 100μg/mL Primocin (InVivogen, ant-pm),

and 5ng/mL heat stable FGF2 (Gibco, PHG0368). After 72 hours, non-adherent tissue was gently removed. Media was changed every 2-3 days thereafter until the culture was 100% confluent. Cells were passaged using CellStripper and plated without gelatin coating. For flow cytometry experiments involving membrane KITL staining, cells were lifted using CellStripper and stained. For other flow cytometry experiments and cytokine stimulation, cells were lifted with Trypsin-EDTA. For LIF stimulation experiments, cells were plated at 5,000 cells/cm2, grown overnight in growth media, then changed to growth media without heat-stable FGF2 with or without 20ng/mL LIF (Peprotech, 250-02). Media was changed after two days and the RNA was harvested on the third day. For TNFα stimulation experiments, cells were plated at 10,000 cell per cm2, grown over night in growth media, then changed to growth media with or without 2.5ng/mL of TNFα (Peprotech, 315-01A). Cells or supernatant were harvested after 24 hours for flow cytometry or ELISA, respectively.

For co-culture with hematopoietic stem and precursor cells, splenic stromal cells were plated and grown until confluence before 5,000 live c-Kit^{+} Lineage^{-} cells were sorted and transferred into individual 24-wells with or without a stromal monolayer. Co-cultures were then grown for 7 days before passage or usage in an experiment as specified. The same media was used for co-culturing as was used for monoculture of splenic stromal cells.

2.4.13 Lentiviral particle production and administration

Murine LIF ORF (NM_008501.2) was purchased from GenScript and cloned into the pCSII-EF1α-IRES2-bsr lentiviral backbone. Lentiviral packaging plasmid psPAX2 (Addgene, plasmid #12260) and VSV-G envelope expressing plasmid PMD2.G (Addgene, plasmid #12259) were gifts from Didier Trono.

293FT cells were transfected with lentiviral DNA using the calcium phosphate method. Virus was concentrated from media using PEG Virus Precipitation Kit (Sigma). Viral titer was determined by QuickTiter™ Lentivirus Associated HIV p24 Titer Kit (Cell Biolabs, INC). Mice were infected by tail vein injection with $4x10^9$ viral particles before sacrifice on day 7 for immunofluorescence experiments or on day 10 for all other experiments.

2.4.14 Immunofluorescence, bright-field, and confocal Microscopy

For immunofluorescence, spleens were removed from animals and directly embedded by freezing into NEG-50 media. 6μm sections were fixed using 4% PFA in PBS then permeabilized in 0.5% Triton-X100 in PBS before blocking with 1% BSA/ 5% donkey serum in PBS. Sections were stained with primary antibodies overnight and then stained with secondary antibodies for 1 hour. Primary antibodies, anti-PDGFRa (AB Online, # ABIN726620) and anti-Ki67 (Biolegend, clone 16A8), were diluted 1:200 for staining. Secondary antibodies were donkey anti-rabbit AF488+ (ThermoFisher, # A32790) and donkey anti-rat AF594+ (ThermoFisher, #A21209). Sections were quenched using ReadyProbes™ Tissue Autofluorescence Quenching Kit (ThermoFisher, R37630) according to manufacturers' instructions before staining with DAPI and mounting with ProLong™ Diamond Antifade Mountant (ThermoFisher, P36970). Slides were sealed and imaged using a Zeiss AxioImager Z2 at the Washington University Center for Cellular Imaging using Zen Blue v.3 for image acquisition and processing. Images were counted manually.

For bright-field microscopy, day 7 stromal:hematopoietic co-cultures were imaged live on an ACCU-SCOPE EXI-600 inverted microscope. Images were processed using ImageJ (*275*).

For confocal microscopy, spleens were removed from animals and fixed in 4% PFA (Electron Microscope Sciences, #15710-S) with PBS for 72hrs. Spleens were washed overnight in PBS and

then sectioned by Vibratome to 300µm. Sections were then cleared using 10% w/v CHAPS and 25% v/v N-Methyldiethanoamine in PBS for 48hrs before washing with PBS followed by 72hrs of blocking 5% donkey serum (Sigma, #D9663) in PBS. Primary antibodies, anti-PDGFRa (AB Online, # ABIN726620), anti-Kitl (R&D, #AB-455-NA) and anti-c-Kit (Biolegend, clone 2B8) were then stained at a 1:200 dilution for 72hrs. Sections were washed with PBS overnight before staining at 1:250 with secondary antibodies, donkey anti-rat AF647+ (Thermo, # A48272), donkey anti-rabbit AF555 (Thermo, A-31572), and donkey anti-Goat AF405+ (Thermo, # A48259). After secondary staining, sections were washed overnight with PBS before dehydration with increasing concentrations of ethanol – 50%, 70%, 95%, and 95% - for at least 2 hours each before incubation with a methyl salicylate solution (Sigma-Aldrich, M6752) for 30-60 minutes in a custom metal chamber with 0.2mm coverslip glass bottom. Tissue sections were then imaged at 1.5µm optical sections using a seven-laser inverted Leica SP8 microscope with full spectral hybrid detectors. All image collection was performed using Leica LAS X software, and analysis was performed using Leica LAS X or Imaris (Bitplane) v8 and v9 software. Images shown are maximum intensity projections of 8 sections representing 10.5µm in depth.

2.4.15 Human tissue datasets and xCell analysis

Transcriptomic data of tumor and normal samples were downloaded from The Cancer Genome Atlas (TCGA), Therapeutically Applicable Research To Generate Effective Treatments (TARGET), and Genotype-Tissue Expression (GTEx) consortiums were downloaded using the UCSC Xena portal (https://xena.ucsc.edu/). Normalized RSEM expected counts were logged for visualization and statistical purposes.

A signature based deconvolution pipeline, xCell (*276*), was used to identify enrichment of stromal populations in the tumor microenvironment. Gene length normalized TPM data from TCGA was downloaded from the UCSC Xena portal was used as an input into xCell for stromal cell deconvolution. Patients were grouped into quartiles by LIF expression and compared across subgroups.

2.4.16 Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA). P values were calculated using unpaired t tests (two-tailed) unless otherwise indicated in the figure legends. P values less than 0.05 was considered statistically significant and displayed above the comparison bars in figures. Each figure represents at least two independent experiments and are presented together unless otherwise specified. Error bars show the standard error of the mean for each sample.

2.4.17 Data sharing statement

The single cell RNA-sequencing data reported in this paper will be uploaded to GEO, and the accession number will be given before time of publication. All data from raw sequencing reads to analyzed data along with the accompanying code where applicable is available to reviewers upon request.

2.5 Results

2.5.1 Murine cancer models have expanded splenic hematopoiesis, often with bias towards myelopoiesis

The MMTV-PyMT mouse is a genetic model of breast cancer where tumors develop in situ due to an oncogene under the control of a promoter expressed primarily in mammary epithelium (*235*). Mice with tumors experience neutrophilia and a drastic increase in spleen weight, spleen cellularity, and splenic HSPCs as measured by c-Kit+/Sca-1+/Lineage- (KSL) and granulocyte-monocyte precursor (GMP) amounts (Fig. 2.1A-E). These changes occur with minimal effects to the splenic common lymphoid progenitors (CLP) (Fig. 2.1F) or the BM compartment (Fig. 2.1G-I). To provide more experimental control than the genetic model, we developed a heterotopic tumor model using a PyMT-B6 cell line derived from tumors of a B6/J syngeneic MMTV-PyMT mouse (*240*). PyMT-B6 tumors in female mice aged 8 to 16 weeks, a gender and age range used in further experimentation unless otherwise stated, produced neutrophilia in animals 21 days post-injection (Fig. 2.2A). Similarly, these mice have increased spleen weight, spleen cellularity, and KSL and GMP amounts, in total and as a percent of CD45+ cells (Fig. 2.2B-G). This effect was not mirrored in the BM compartment or the splenic common lymphoid progenitors (Fig. 2.2H-K). Additionally, increased CFU-GEMM colony numbers were identified in the PB of PyMT-B6 bearing animals compared to controls (Fig. 2.3A). Together, data from both a genetic and transplantation model identify the spleen as a site of profound HSPC expansion coincident with increased granulocytes and primitive hematopoietic precursors in the PB.

Enhanced survival of irradiated Ly5.2 mice transplanted with Ly5.1 splenocytes of PyMT-B6 bearing animals compared to mice receiving control splenocytes validated the stem cell functional

of these splenic HSPCs (Fig. 2.3B). Furthermore, animals that survived the initial 28 days continued to live after four months, had reconstitution of PB myeloid and lymphoid lineages, and < 1% contribution from recipient HPSCs in the PB. Splenectomized animals had reduced PMN percentages in their PB after 21 days of PyMT-B6 tumor compared to sham surgery controls (Fig. 2.3C). To generalize our findings about expanded splenic hematopoiesis to other cancer models, heterotopic models of 1956 sarcoma and LLC lung carcinoma were investigated and found to significantly expand HSPC and GMP populations with a variable response to peripheral neutrophilia (Fig. 2.4A-F). Together, these data indicate that breast cancer induces expansion of splenic hematopoiesis that is necessary for neutrophilia and this expanded capacity is generalizable to other murine cancer models.

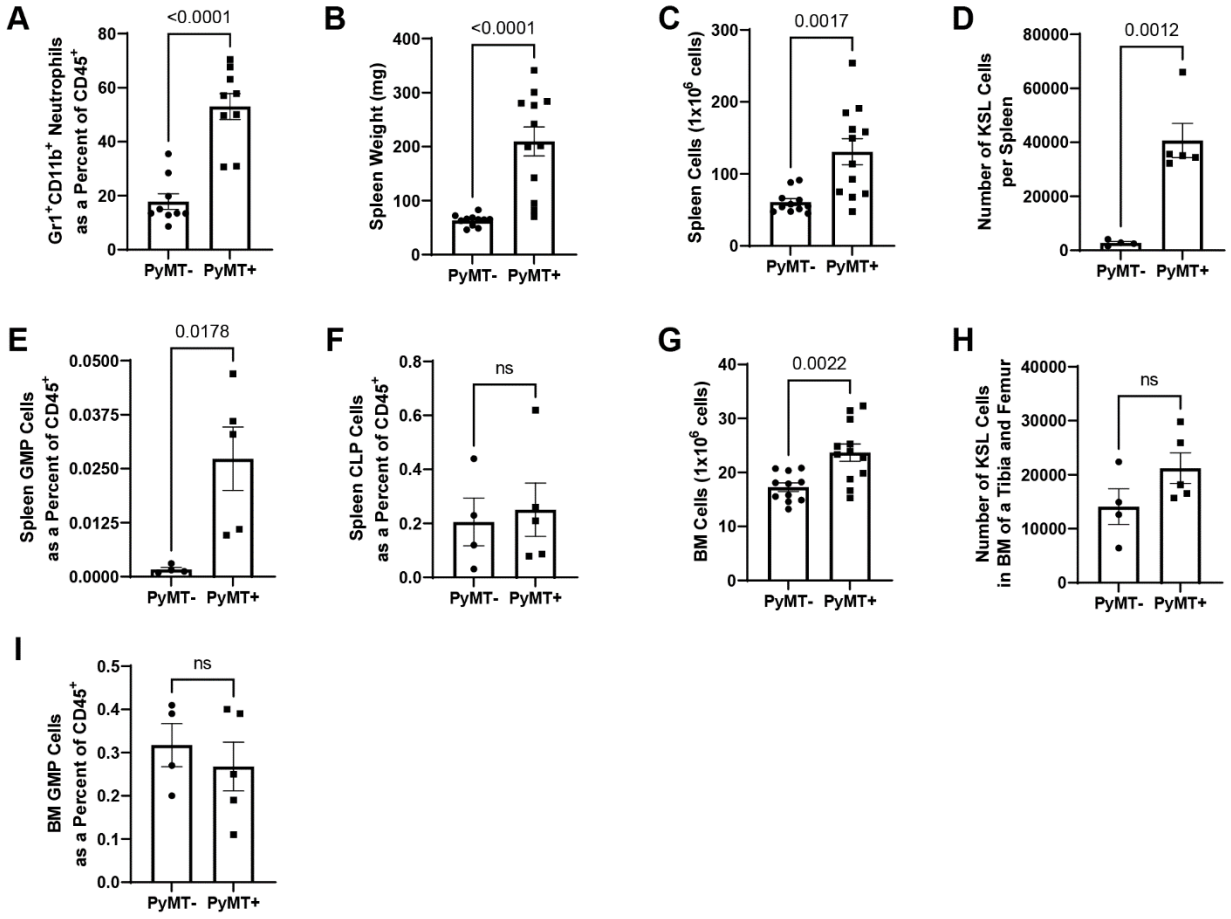

Figure 2.1: Genetic PyMT tumors induce splenic hematopoiesis

(A) PMNs in the peripheral blood as percent of $CD45^+$ cells in female mice between the ages of 3 to 6 months with spontaneous mammary tumors in the MMTV-PyMT tumor model compared with non-tumor bearing, littermate controls (n = 9). (B – I) In mice with MMTV-PyMT mammary tumors compared with littermate controls, splenic weight (n = 11-12), splenic cellularity (C, n = 11-12), KSL cells per spleen (D, n = 4-5), GMP cells as a fraction of total splenic $CD45^+$ (E, n = 4-5), CLP cells as a fraction of total splenic $CD45^+$ cells (F, n = 4-5), BM cellularity per leg (G, n = 11-12), KSL cells per BM of a leg (H, n = 4-5), GMP cells as a fraction of total BM CD45+ cells (I, n = 4-5).

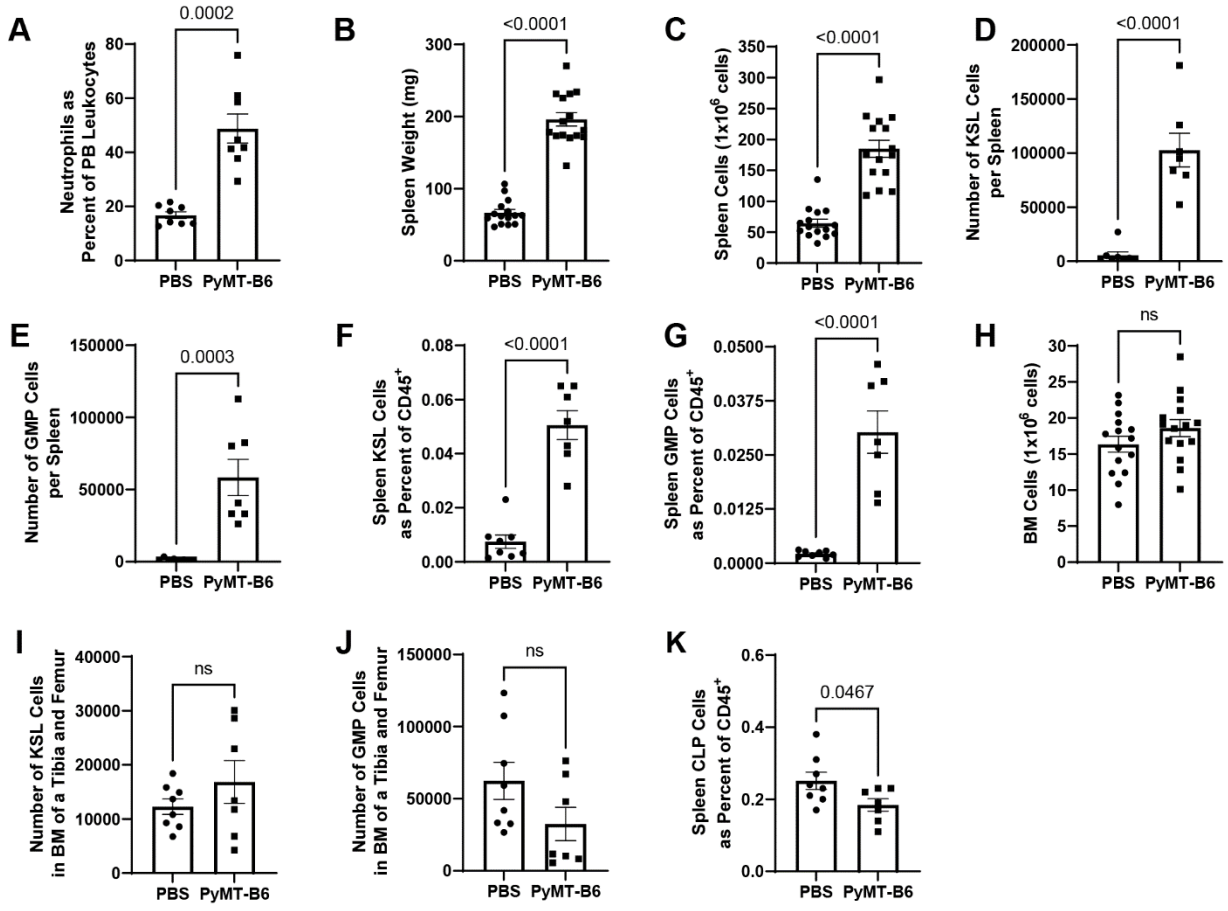

Figure 2.2: Heterotopic PyMT-B6 tumors induce splenic hematopoiesis

(A - K) 21 days after injection of 5 x 10^5 PyMT-B6 tumor cells injected subcutaneously compared to control animals injected with phosphate buffered saline, PMNs in the PB as a percent of total leukocytes (A, n = 8), splenic weight (B, n = 15), splenic cellularity (C, n = 15), KSL cells per spleen (D, n = 7-8), GMP cells per spleen (E, n = 7-8), KSL cells as a fraction of total splenic $CD45^+$ cells (F, n = 7-8), GMP cells as a fraction of total splenic $CD45^+$ cells (G, n = 7-8), BM cellularity per leg (H, n = 15), KSL cells per BM of a leg (I, n = 7-8), BM GMP cells per BM of a leg (J, n = 7-8), CLP cells as a fraction of total splenic $CD45^+$ cells (K, n = 7-8).

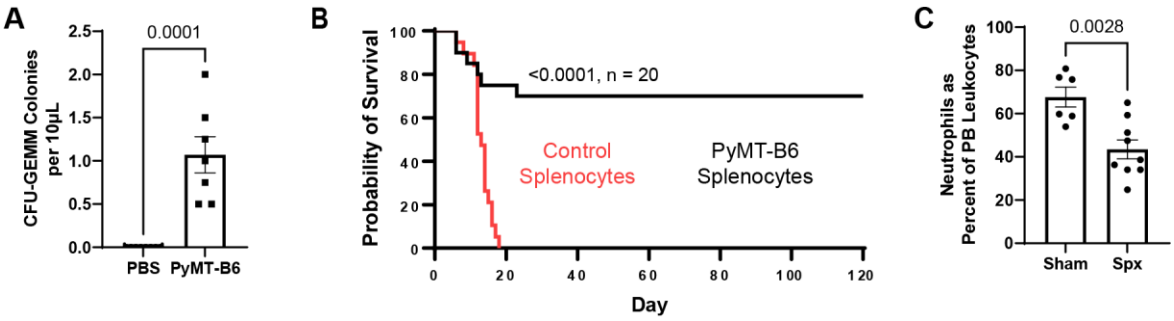

Figure 2.3: Expansion of splenic hematopoiesis is required for neutrophilia in PyMT-B6

(A) CFU-GEMM colonies within 10µL of PB, 21 days of PyMT-B6 tumor compared to PBS injected controls (n = 7-8). (B) Survival of 9.5 Gy irradiated mice receiving splenocytes from mice with 21 days of PyMT-B6 tumor or control mice injected with PBS (n = 20, significance assigned by Mantel-Cox test). (C) PMNs in the PB as a percent of total leukocytes with 21 days of PyMT-B6 tumor following splenectomy or sham surgery (n = 7-8).

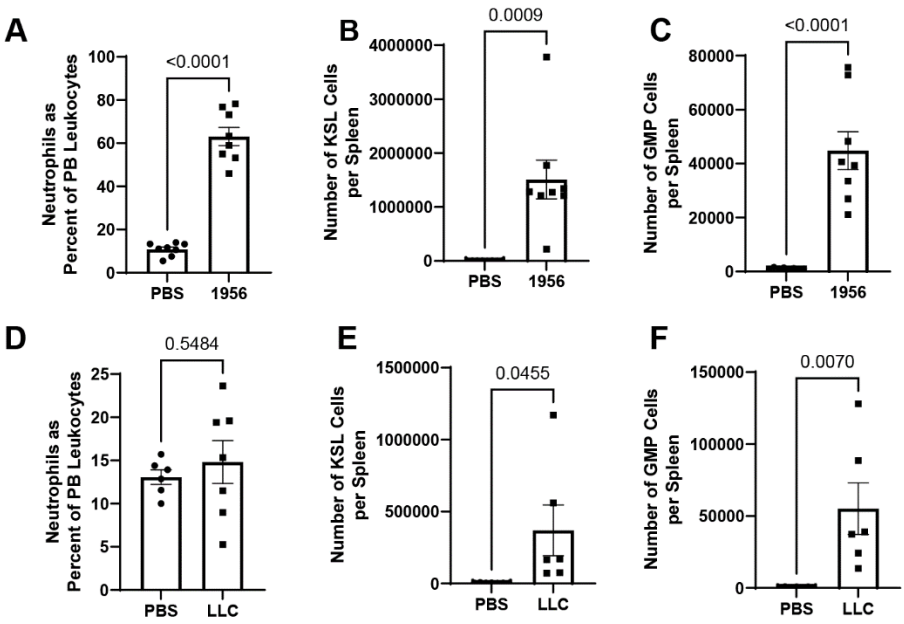

Figure 2.4: Heterotopic 1956 and LLC tumors induce splenic hematopoiesis

(A – C) 17 days after injection of 2 x 10^6 1956 tumor cells injected subcutaneously compared to control animals injected with phosphate buffered saline, PMNs in the PB as a percent of total leukocytes (J, n = 8), KSL cells per spleen (B, n = 8), GMP cells per spleen (K, n = 8). (D – F) 16 days after injection of 5 x 10^5 LLC tumor cells injected subcutaneously compared to control animals injected with phosphate buffered saline, PMNs in the PB as a percent of total leukocytes (D, n = 6-7), KSL cells per spleen (E, n = 6-7), GMP cells per spleen (F, n = 6-7).

2.5.2 HSPCs conducting EMH express an inflammatory gene profile

To characterize the transcriptional and cell composition changes induced by the PyMT-B6 tumor, we performed single-cell RNA-sequencing (scRNA-seq) of splenic and BM cells enriched for HSPCs in mice with or without PyMT-B6 tumor. The $Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/CD34^+$ cells representing the hematopoietic stem and progenitor cells (Fig. 2.5A-H, Fig. 2.6A-C), while rarely present in the control spleen, were well-represented in the spleen of the tumor-bearing mice (Fig. 2.6D). The $Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/CD34^+$ cells from the spleen of tumor-bearing animals expressed a unique gene

signature, including *Tnf*, *Cxcl2*, *Nfkbiz*, *Nfkbia*, compared to control BM (CBM) cells or tumor-bearing BM (TBM) cells (Fig. 2.6E-H). We chose to focus on the comparison between control bone marrow and HPSCs associated with EMH in the spleen to address questions about the functional differences between homeostatic HSPCs and those associated with pathology and residing in extramedullary sites. The up-regulation of these four genes in tumor-bearing, spleen (TS) $Lin^-/CD34^+$ HSPC compared to the same population in the homeostatic BM was confirmed by RT-qPCR (Fig. 2.7A-D). Increased TNFα protein expression was identified within the HSPC fraction of TS relative to CBM by flow cytometry (Fig. 2.7E-G). Together, these data demonstrate that PyMT-B6 tumor presence activates an inflammatory gene signature within splenic HSPCs that induces TNFα production by these cells.

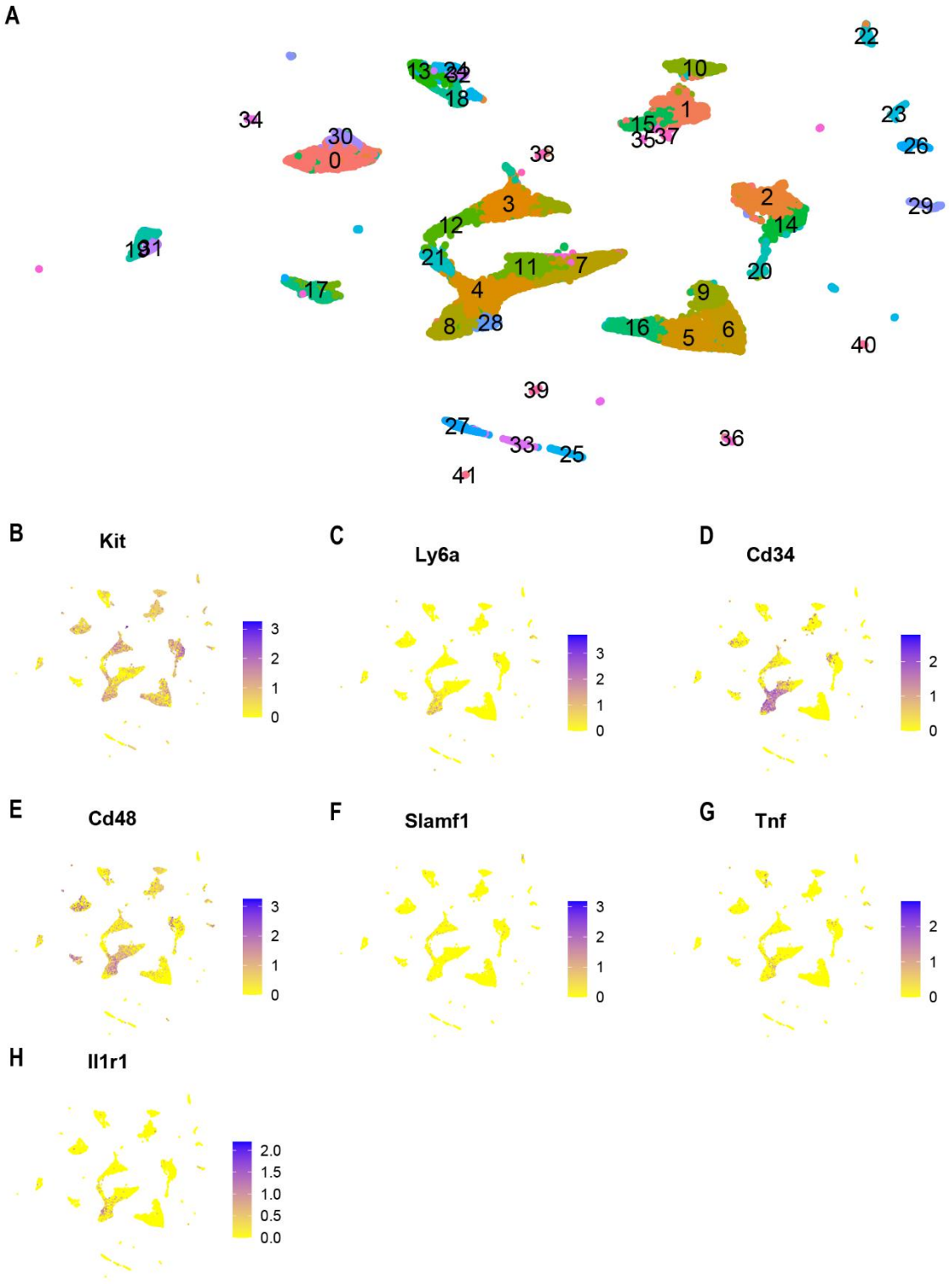
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Figure 2.5: c-Kit^{+}Sca-1^{+}CD34^{+} HSPCs can be found within the bone marrow and spleen of mice with or without PyMT-B6 tumors.

(A – H) UMAP projection of the total scRNA-seq data colored by cluster identity (A), expression of *Kit* (B), *Ly6a* (C), *Cd34* (D) , *Cd48* (E) , Slamf1 (F) , *Tnf* (G) , *Il1r1* (H)

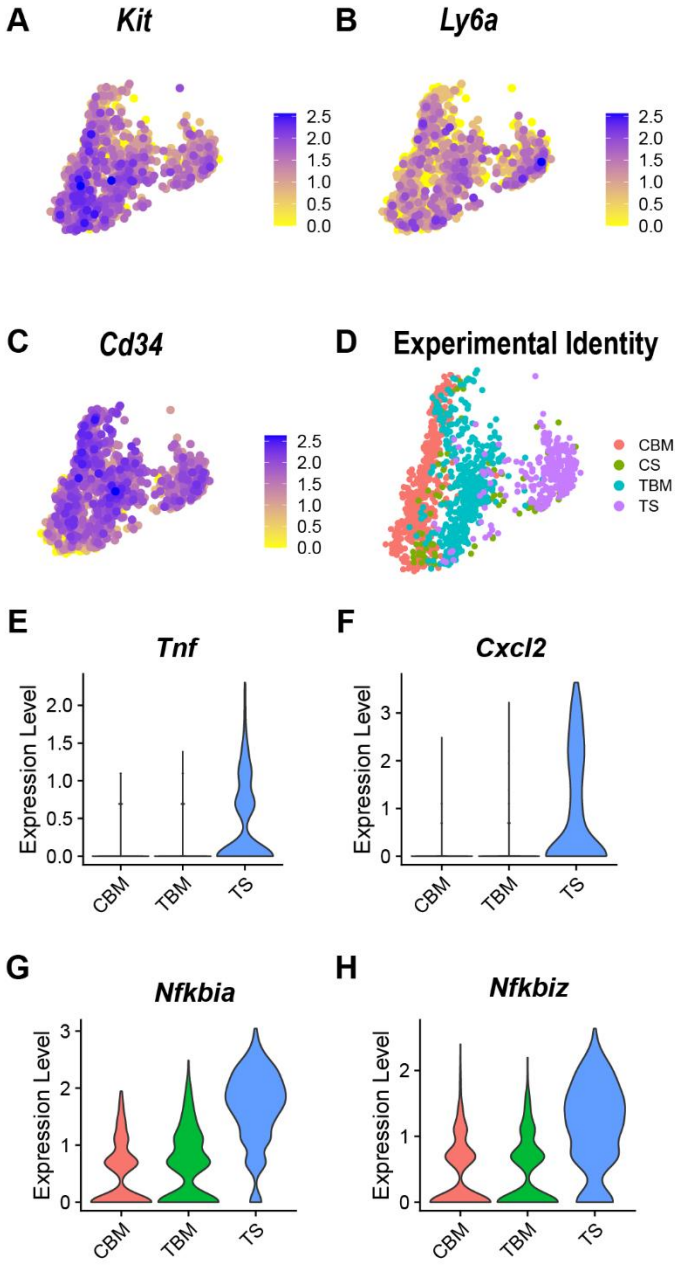

Figure 2.6: HSPCs from tumor-bearing mice display an inflammatory gene signature

(A – D) UMAP projection of the HSPC population in scRNA-seq data colored by expression of *Kit* (A), *Ly6a* (B), *Cd34* (C) and by experimental origin (D, CBM – Control BM, CS – Control Spleen, TBM – Tumor-bearing BM, TS – Tumor-bearing spleen). (E – H) Violin plot of expression of *Tnf* (E), *Cxcl2* (F), *Nfkbia* (G), and *Nfkbiz* (H) in the HSPC population in scRNA-seq data from CBM, TBM, or TS.

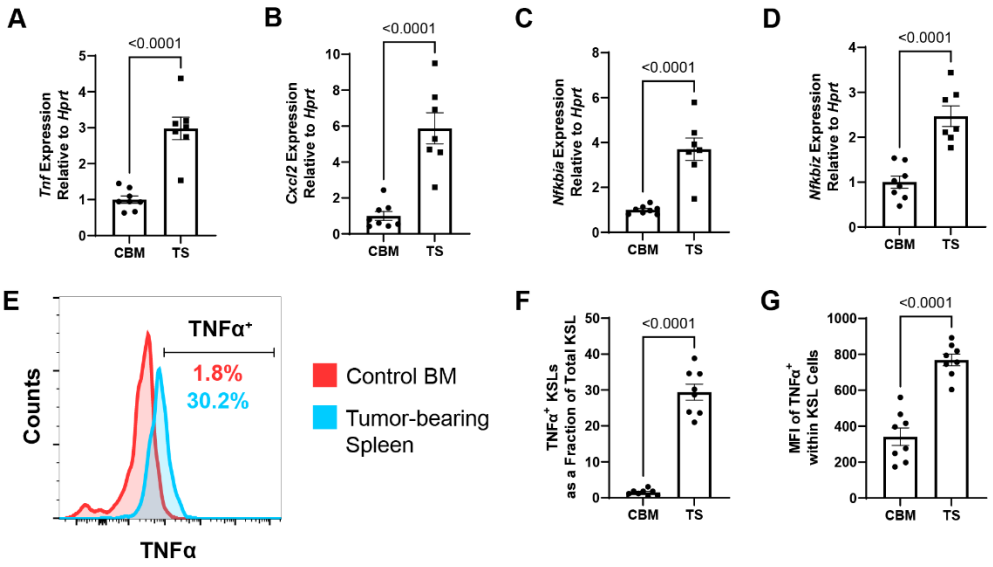

Figure 2.7: Inflammatory gene profile in HSPCs from tumor-bearing mice can be validated by RT-qPCR and flow cytometry

(A - D) RT-qPCR expression data of *Tnf* (A), *Cxcl2* (B), *Nfkbia* (C), *Nfkbiz* (D) from $Lin^-/Flk1^-/CD34^+$ cells from CBM or TS. (E) Representative histogram of TNFα expression in KSL cells from CBM or TS. (F) Fraction of KSL cells from CBM or TS that are $TNF\alpha^+$ (n = 8). (G) Mean fluorescent intensity of TNFα staining in KSL cells from CBM and TS (n = 8).

2.5.3 PyMT-B6-produced IL-1α acts on HSPCs to induce TNFα

TNFα expression in splenic HSPCs of PyMT-B6 bearing mice hints at the presence of tumor-derived upstream mediators. One often reported cytokine subfamily upstream of TNFα is IL-1α and IL-1β (*277*); of which, IL-1α, but not IL-1β, is produced during MMTV-PyMT tumor pathology (*278*). We confirmed that mice bearing PyMT-B6 tumors have elevated circulating levels of IL-1α (Fig. 2.8A) and that PyMT-B6 cells release IL-1α in vitro (Fig. 2.8B). Correspondingly, IL-1 receptor was identified as being expressed in HSPCs by scRNA-seq and RT-qPCR (Fig. 2.8C-D). Injection of IL-1α into mice was sufficient to induce neutrophilia, increase splenic HSPC fraction, and increase TNFα production in splenic HSPCs compared to control BM HSPCs (Fig. 2.9A-C). Reciprocally, deletion of Il1a from PyMT-B6 cells led to decreased expression of TNFα in HSPCs and decreased total splenic GMP cells compared the parental line (Fig. 2.10A-D). The parental line includes deletion of G-CSF due to the cytokines recognized prominence in producing myeloid-biased hematopoiesis and EMH (*279*). Removing this cytokine was designed to uncover effects of other cytokines produced by the tumor, such as IL-1α. Together, these data indicate that PyMT-B6 IL-1α induces a novel inflammatory phenotype in HSPCs associated with tumor-induced EMH.

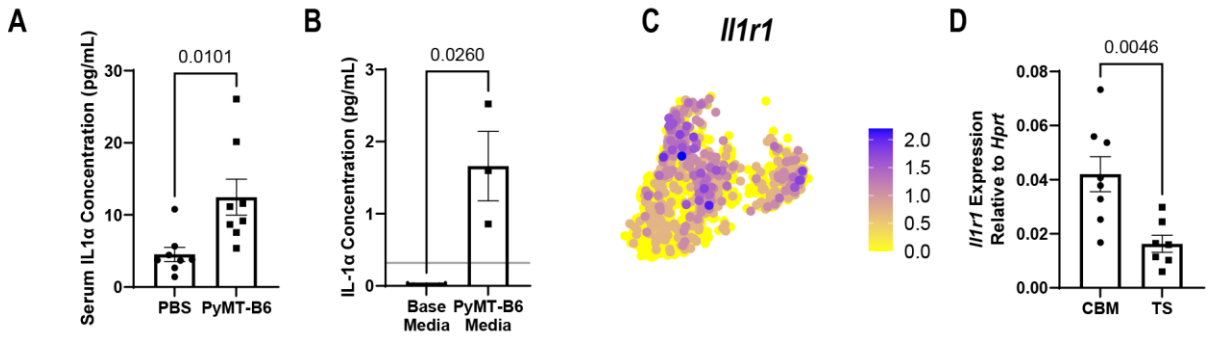

Figure 2.8: PyMT-B6 tumors produce IL-1α and HSPCs express the IL-1 receptor

(A) IL-1α concentration from serum of mice with or without 21 days of PyMT-B6 tumor (n = 8). (B) IL-1α concentration from PyMT-B6 base or conditioned media (n = 3). (C) UMAP projection of HSPCs in scRNA-seq data colored by *Il1r1* expression. (D) RT-qPCR expression data of *Il1r1* from $Lin^-/Flk1^-/CD34^+$ cells from control bone marrow (CBM) or PyMT-B6 tumor bearing spleen (TS).

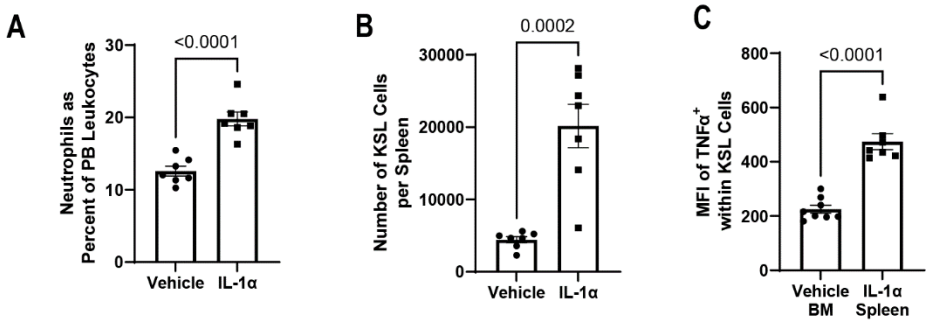

Figure 2.9: IL-1α is sufficient to activate EMH and TNFα production in splenic HSPCs

(A – B) In mice 24 hours after intravenous (i.v.) injection of 500ng IL-1α or vehicle, PMNs in the PB as a percent of total leukocytes in mice (A, n = 7), KSL cells per spleen, (B, n = 7). (C) Average mean fluorescent intensity of TNFα staining in KSL cells from vehicle injected BM or 500ng IL-1α injected spleen. (n = 7-8).

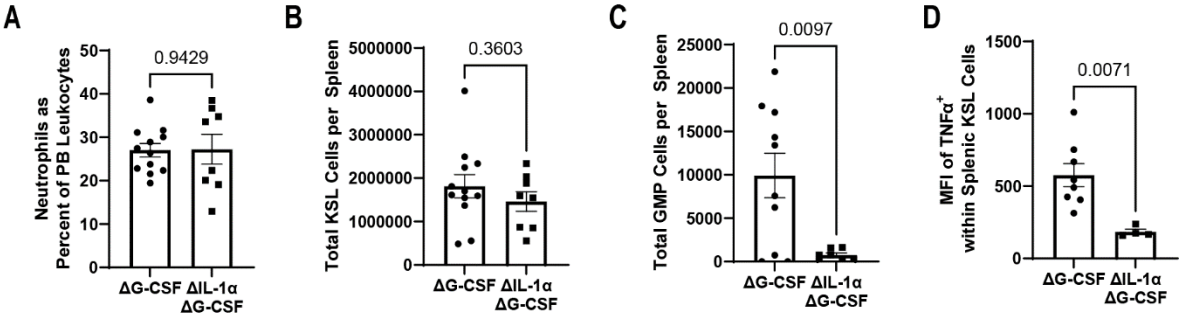

Figure 2.10: Tumor-derived IL-1α is required for increased splenic myelopoiesis and TNFα production in splenic HSPCs

(A – D) 28 days after subcutaneous injection of 2.5 x 10^5 PyMT-B6 ΔG-CSF parental cells or ΔG-CSF ΔIL-1α cells, PMNs in the PB as a percent of total leukocytes in mice (A, n = 7-13), KSL cells per spleen, (B, n = 7-13), GMP cells per spleen (C, n = 7-13), average mean fluorescent intensity of TNFα staining in KSL cells (D, n = 7-13).

2.5.3 TNFα induces EMH through splenic niche cells

Due to the concurrent TNFα production by splenic HSPCs and splenic EMH accompanying PyMT-B6 tumors, we hypothesized that TNFα from HSPCs could induce EMH by activating local niche cells. Administration of a single dose of TNFα was sufficient to increase HSPC and GMP fractions in the spleen within 24 hours and to produce neutrophilia (Fig. 2.11A-C). To test whether niche cells respond to TNFα, we needed to identify potential niche cells within the spleen. Reanalysis of a BM niche cell scRNA-seq dataset (*274*) identified Pdgfra+/Pdgfrb+ stromal (ABS) cells as being the most strongly KIT ligand positive cell population and expressing a TNFα receptor (cluster 3, Fig. 2.12A-L). Using a novel method of cell isolation and culture, we cultured ABS cells from the spleen and validated their expression of membrane KIT ligand by flow cytometry (Fig. 2.13A-B). To investigate the niche functionality of these ABS cells, 5,000 live BM Lin-/c-Kit+ (KL) cells, of which around 20% were also Sca-1+, were sorted into 24-wells with or without confluent ABS cell cultures. After 7 days of co-culture, a large population of small, spherical cells grew on top of the ABS monolayer (Fig. 2.13C-D). Upon flow cytometric evaluation, these co-cultures contained a population of $CD45^+/Lin^-/c\text{-}Kit^+/Sca\text{-}1^+$ cells (Fig. 2.13E). To test whether the hematopoietic component of these co-cultures maintained stem cell capacity, first, CD45.1 KL cells were sorted into plates and grown for 7 days with or without ABS stromal before transplanting them into irradiated CD45.2 recipient mice. Compared to mice receiving KL cells grown without ABS cells, mice that received KL cells grown with ABS cells

had significantly improved survival, indicating the maintenance of repopulating units in vitro (Fig. 2.14A). Analysis of the peripheral blood from surviving transplant mice indicated recipient derived hematopoietic cells constituted less than 10% of all CD45+ cells after one month. Second, colony forming unit activity was compared between KL cells grown with or without ABS cells for 7 days. After 7 days, more primitive precursor activity, as measured by CFU-GEMM colony formation, was nearly absent from cells without co-culture but preserved in cells grown in co-culture (Fig. 2.14B). Additionally, CFU-GEMM colonies were observed until at least 21 days in co-culture. Next, we wanted to understand how ABS cells might change phenotypically in response to HSPC cytokines. Following TNFα addition to culture medium, splenic ABS cells increased HSPC-adherent VCAM-1 expression and released the HSPC active cytokine CXCL1 while maintaining baseline CXCL12 release (Fig. 2.15A-C). Together, these data suggest that TNFα produced by HSPCs during tumor presence can act locally on ABS niche cells to increase the capacity of the splenic niche to support hematopoiesis.

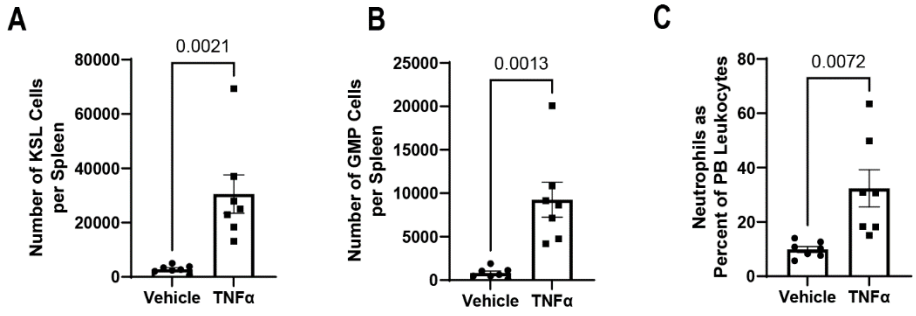

Figure 2.11: Tumor-derived IL-1α activates TNFα production in splenic HSPCs

(A – C) In mice 24 hours after i.v. injection of 2μg TNFα or vehicle, KSL cells per spleen (A, n = 7), GMP cells per spleen (B, n = 7), PMNs in the PB as a percent of total leukocytes (C, n = 7).

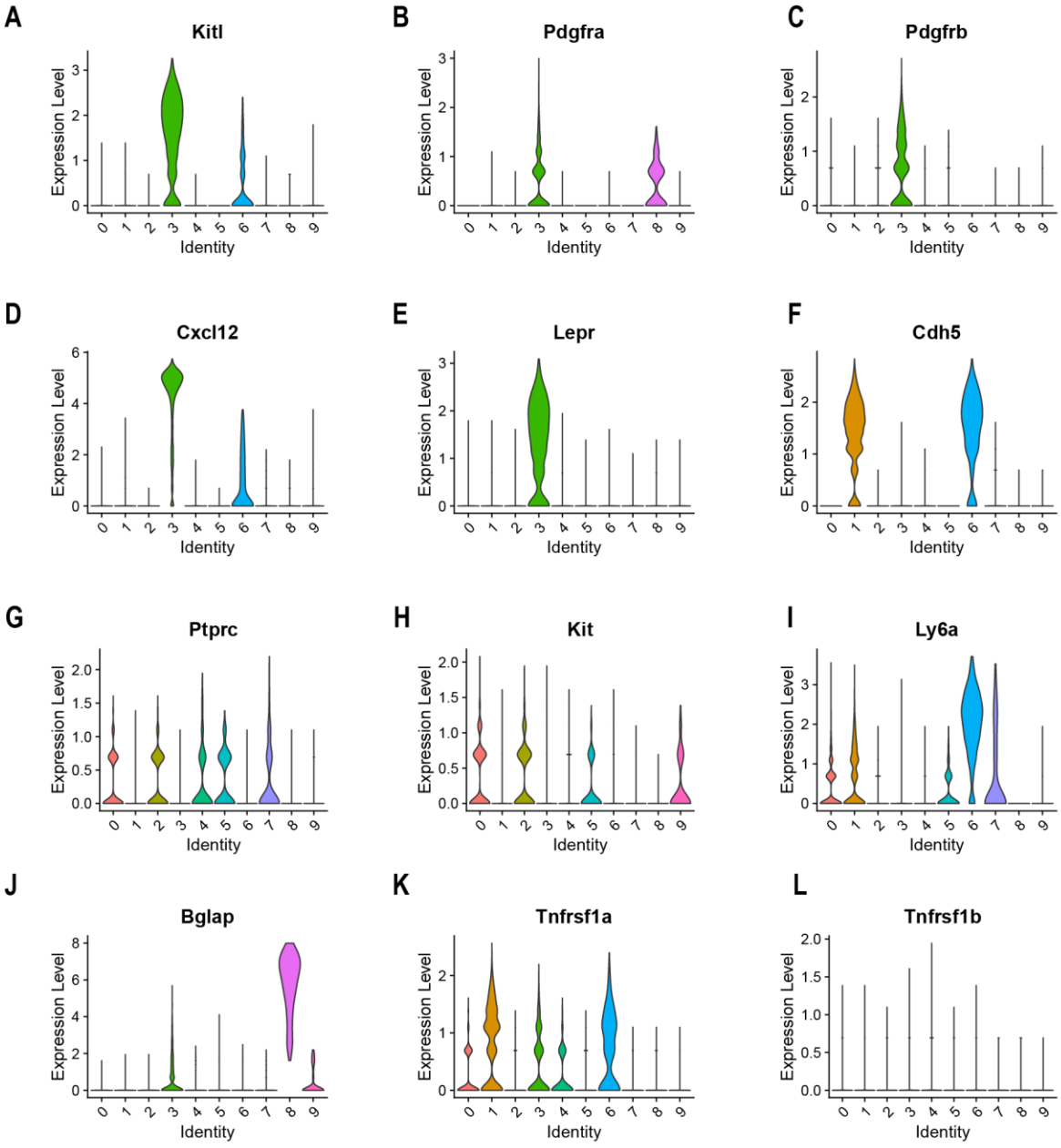

Figure 2.12: Expression patterns of bone marrow niche cells by single cell RNA-sequencing

(A – L) Violin plot of expression of *Kitl* (A), *Pdgfra* (B), *Pdgfrb* (C), *Cxcl12* (D), *Lepr* (E), *Cdh5* (F), *Ptprc* (G), *Kit* (H), *Ly6a* (I), *Bglap* (J), *Tnfrsf1a* (K), *Tnfrsf1b* (L), in reanalyzed scRNA-seq data from Tikhonova *et al.* 2019 of bone marrow niche cell types (0 – HSC, 1 – endothelium (EC), 2 – Proliferating $CD45^{+}$, 3 – ABS cell, 4 – GMP, 5 – CLP, 6 – Sca-1^{+} EC, 7 – B-cell progenitor, 8 – Osteoblast, 9 – RBC Progenitor) (*274*).

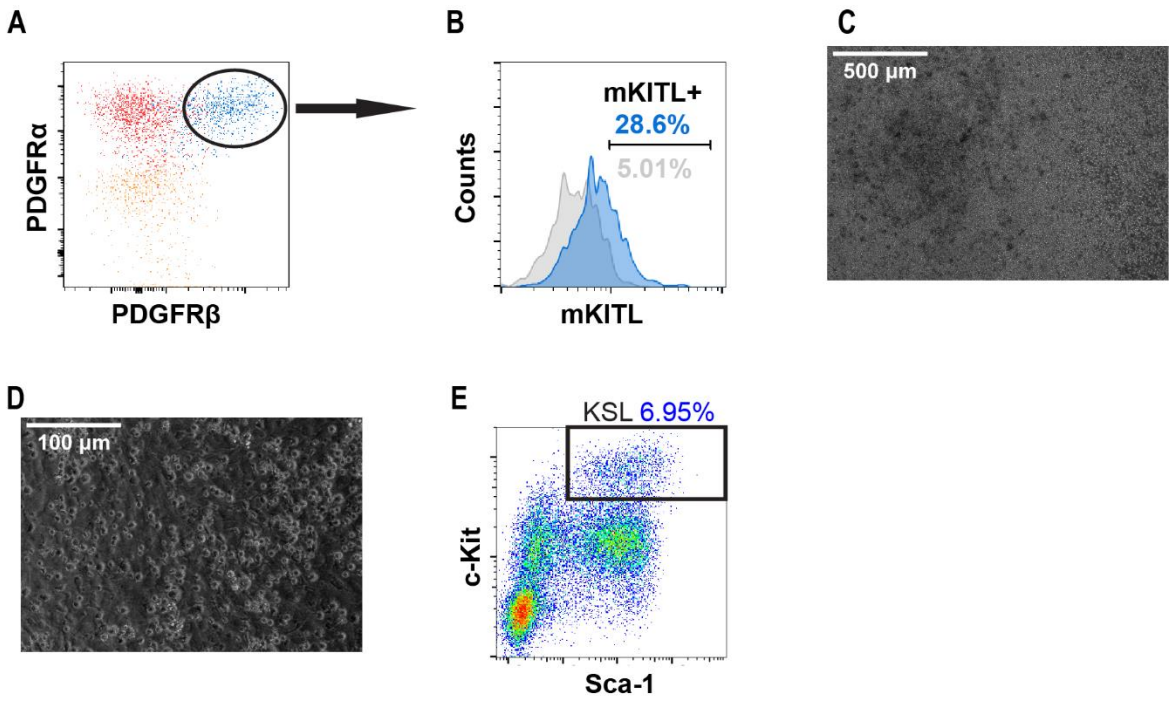

Figure 2.13: Identification of splenic stromal cells analogous to bone marrow niche populations that can maintain hematopoietic cells in co-culture

(A) Representative dot plot comparing splenic ABS cells stained solely for viability, a PDGFRβ fluorescence minus one sample, and a fully stained sample. (B) Representative histogram of membrane KITL expression in splenic ABS cells. (C – E) Hematopoietic and stromal co-cultures after 7 days, representative bright-field image of co-culture at 4x magnification (C), representative bright-field image of co-culture at 20x magnification (D), representative flow cytometric plot of Live/CD45^{+}/Lin^{-} cells (E).

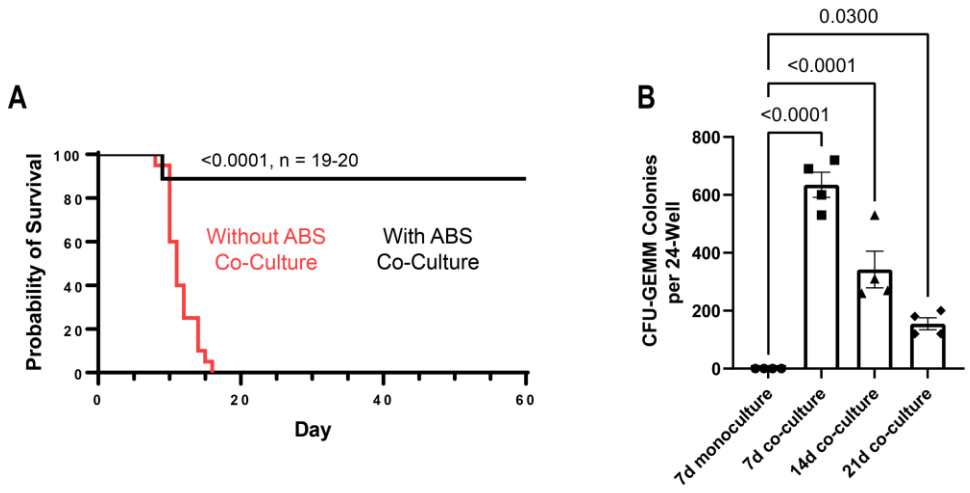

Figure 2.14: Hematopoietic cells in co-culture with splenic stromal cells maintain progenitor status

(A) Representative histogram of membrane KITL expression in splenic ABS cells. (B) Survival of 9.5 Gy irradiated mice receiving the cell products of c-Kit^{+}Lin^{-} cells grown with or without ABS cell co-culture for 7 days (n = 19-20, significance assigned by Mantel-Cox test).

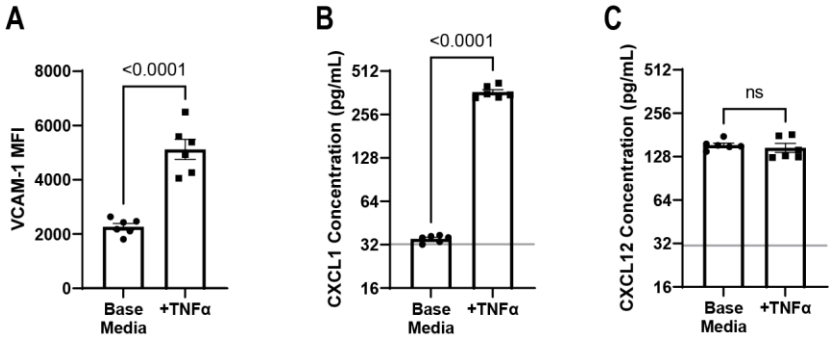

Figure 2.15: TNFα activates stromal cells in the spleen to induce EMH

(A – C) In splenic ABS cells treated for 24 hours with or without 2.5ng/mL TNFα, representative mean fluorescent intensity of VCAM-1 (A, n = 6), representative CXCL1 concentration (B, n = 6), representative CXCL12 concentration (C, n = 6).

2.5.4 Tumor-derived Leukemia Inhibitory Factor activates splenic EMH

Given the indirect interaction between tumor cells and splenic niche cells through inflamed HSPCs, we were interested in the potential of a direct interaction between tumor and splenic niche cells. Preliminary analysis of a 44-member cytokine array on serum from MMTV-PyMT tumor-bearing animal compared to littermates identified Leukemia Inhibitory Factor (LIF), an IL-6 family member, as being significantly and consistently upregulated by the presence of tumors (Fig. 2.16). The presence of LIF in the serum of PyMT-B6 bearing animals and the production of LIF by PyMT-B6 cells in culture was independently confirmed (Fig. 2.17A-B). Previous work has identified LIF as having an active role in promoting and maintaining hematopoiesis in the spleen (*180, 280*). We tested whether LIF might have a role in cancer-induced EMH by generating a lentiviral expression vector for murine LIF and injecting mice intravenously to induce systemic LIF overexpression. Compared to empty lentiviral vectors, LIF overexpression induced neutrophilia and a robust expansion of HSPC and GMP cells in the spleen (Fig. 2.17C-E). Correspondingly, deletion of *Lif* from PyMT-B6 cells lead to decreased levels of splenic HSPCs and GMPs compared to the G-CSF deleted parental line (Fig. 2.18A-C). These data identify LIF as a tumor-secreted factor which is sufficient to induce myeloid-biased expansion of hematopoiesis within the spleen.

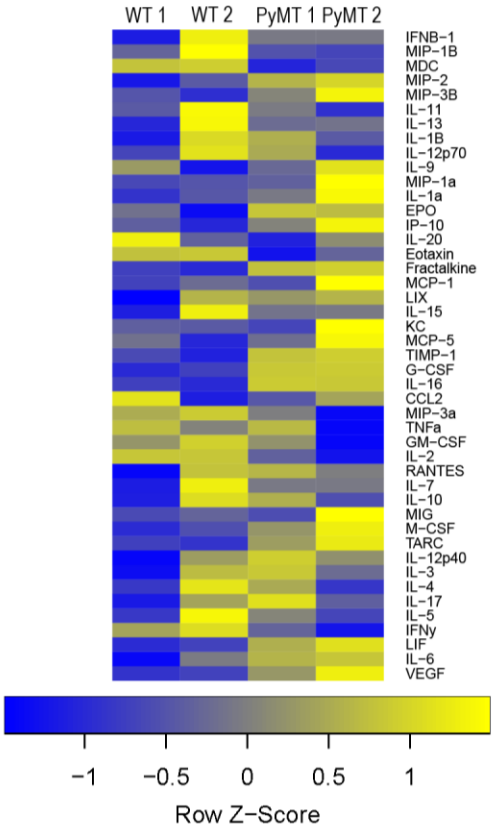

Figure 2.16: Serum cytokine profiling of MMTV-PyMT tumor bearing mice identifies LIF overexpression

Heatmap showing z-scores of log normalized expression of 44 cytokines from the serum of MMTV-PyMT^{+}, tumor-bearing animals (PyMT) or age-matched, non-tumor-bearing littermates (WT) (n = 2).

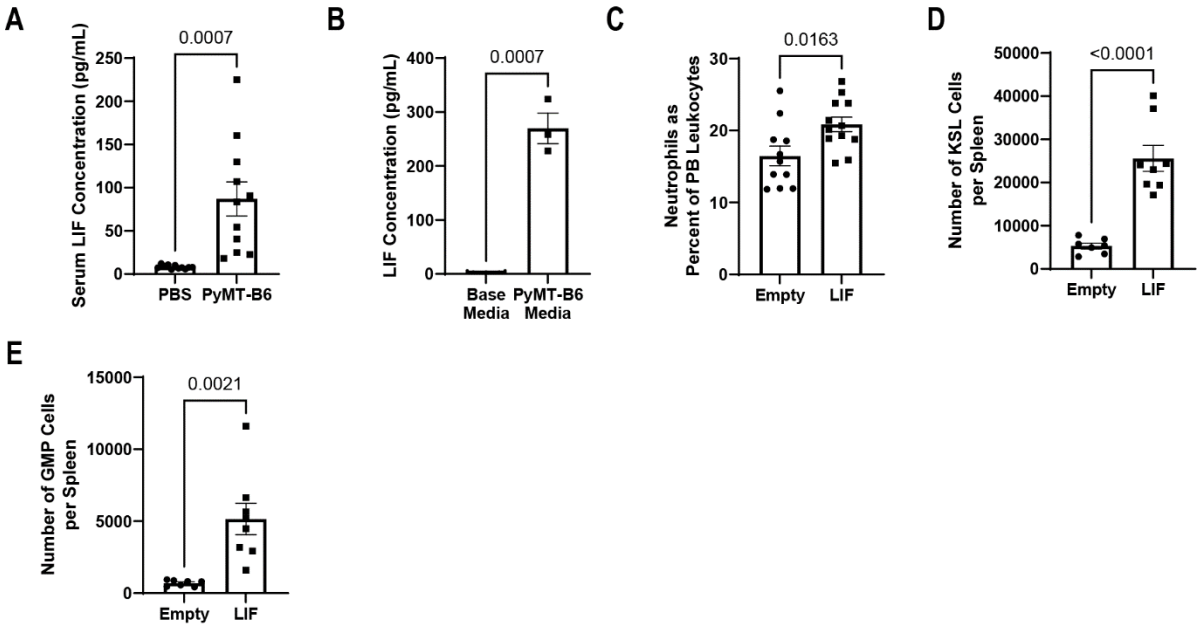

Figure 2.17: Tumor-produced Leukemia Inhibitory Factor induces EMH

(A) LIF concentration from serum of mice with or without 21 days of PyMT-B6 tumor (n = 11). (B) LIF concentration from base media or PyMT-B6 conditioned media (n = 3). (C – E) In mice with 10 days of LIF overexpression or empty vector control, fraction of PMNs in the PB as a percent of total leukocytes (C, n = 11-12), KSL cells per spleen (D, n = 7-8), GMP cells per spleen (E, n = 7-8)

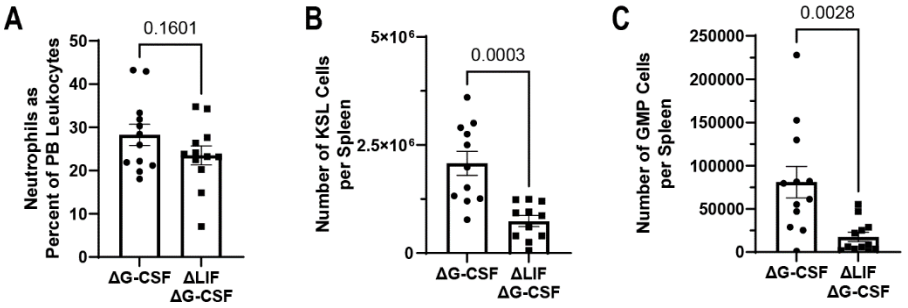

Figure 2.18: *Lif* deletion in PyMT-B6 cells reduces tumor induced EMH

(A – C) 28 days after subcutaneous injection of 2.5 x 10^5 PyMT-B6 ΔG-CSF parental cells or ΔG-CSF ΔLIF cells, PMNs in the PB as a percent of total leukocytes in mice (A, n = 12), KSL cells per spleen (B, n = 12), GMP cells per spleen (C, n = 12).

2.5.5 Leukemia Inhibitory Factor induces splenic stromal niche cell proliferation

Having identified the capability of LIF to expand splenic hematopoietic capacity, we sought to define a cellular mechanism for its effect. Re-examination of niche scRNA-seq data (*274*) identified both Kitl expressing clusters, Cdh5+/Ly6a+ endothelial and Pdgfra+/Pdgfrb+ stromal cells, as expressing LIF receptor (LIFR) (Fig. 2.19A, cluster 6 vs 3, respectively). By inducing LIF overexpression by lentivirus in Cdh5-Cre^{+}/$Lifr^{fl/fl}$ mice and littermate controls, we could exclude endothelial cell contribution to LIF-induced EMH (Fig. 2.19B). We were unable to directly test the involvement of splenic ABS cells in response to LIF because mice with LIFR deletion within the PDGFRα+ population were born at normal frequencies but died before weaning due to a failure to thrive, a similar but less severe phenotype than the constitutive knock out mouse (Fig. 2.19C-D) (*281*). At days 12 post-partum, when most conditional knockouts are still alive and the spleen still shows active hematopoiesis (*282*), we assessed whether deletion of LIFR with PDGFRα+ cells affected hematopoietic capacity of the spleen. We found that mice with LIFR deletion in PDGFRα+ had reduced HSPCs specifically within the spleen compared to the bone marrow and littermate controls (Fig. 2.19E-F). This suggests that the LIF-LIFR axis in PDGFRα+ cells is indispensable for maintenance of hematopoiesis specifically within the spleen, and therefore we were interested in potential mechanisms. Previous studies showed that LIF induces proliferation of PDGFRα+ oligodendrocyte precursor cells and osteoblast precursors (*199, 283*). We added LIF to splenic ABS cultures and found increased markers of proliferation (Fig. 2.20A). To confirm this

finding in vivo, we quantified the fraction of Ki67+ nuclei of PDGFRα+ cells in the spleen with or without lentiviral LIF overexpression using immunofluorescence and found an increase in Ki67+ PDGFRα+ cells with LIF overexpression (Fig. 2.20B-C). Additionally, we used confocal imaging to visualize the close association of PDGFRα+ cells with Kit+ progenitors in the spleen after LIF overexpression (Fig. 2.20D).

To investigate whether this expansion of stromal cells might operate within human cancers, we analyzed RNA-sequencing data from a collection of sources to assess whether LIF expression in human cancer correlates with local stromal cell populations (*284*). Consistent with our mouse data, tumors in the highest quartile of LIF expression had significantly higher amounts of MSCs, fibroblasts, and stromal scores compared to the lowest quartile with only a modest increase in the endothelial fraction between the two groups (Fig. 2.21A-D). Together, these data suggest that ABS cells from the spleen form an expandable niche in the spleen in direct response to tumor-derived LIF and that this cancer-stromal interaction may operate in human tumors as well.

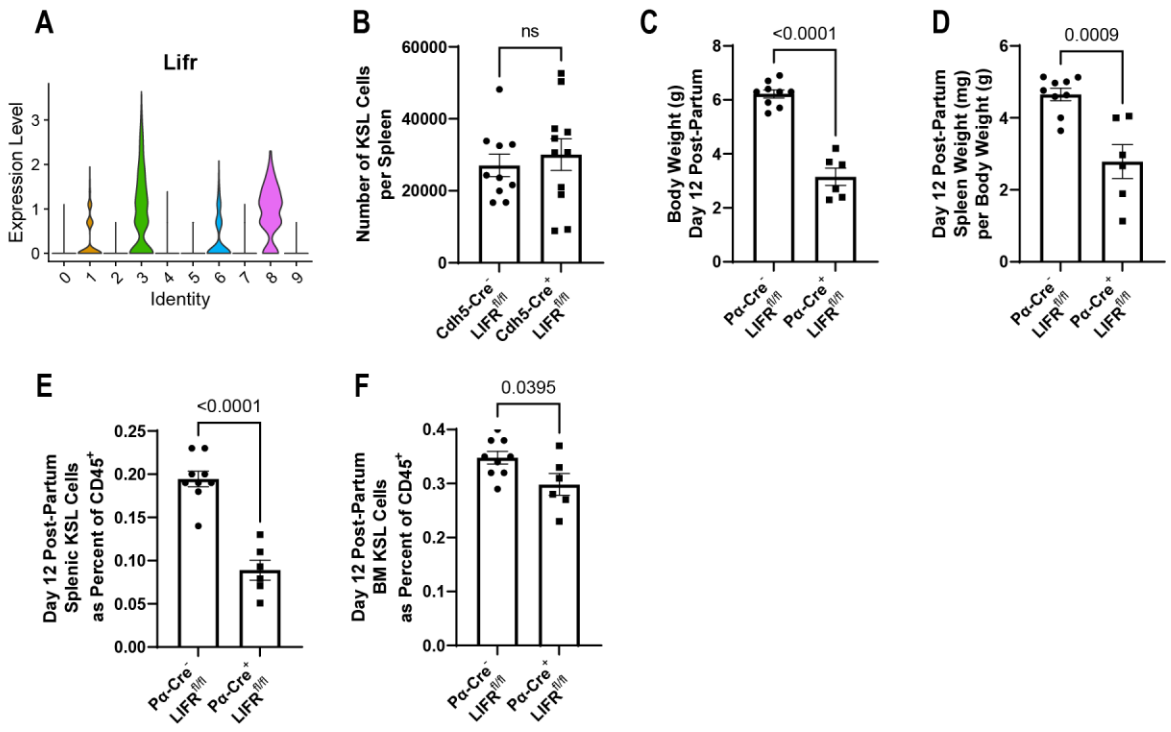

Figure 2.19: LIF receptor is expressed in stromal and endothelial cells and is required in stromal cells for splenic hematopoiesis

(A) Violin plot of expression of *Lifr* in reanalyzed scRNA-seq data from Tikhonova *et al.* 2019 of bone marrow niche cell types (0 – HSC, 1 – endothelium (EC), 2 – Proliferating CD45$^+$, 3 – ABS cell, 4 – GMP, 5 – CLP, 6 – Sca-1$^+$ EC, 7 – B-cell progenitor, 8 – Osteoblast, 9 – RBC Progenitor) (*274*). (B) In LIFR^{flox} mice with LIF overexpression and Cdh-Cre$^+$ or Cdh5-Cre$^-$, KSL cells per spleen (n = 10-11, contains male mice). (B – F) In day 12 post-partum LIFR^{flox} mice with PDGFRα-Cre$^+$ or PDGFRα-Cre$^-$ littermates, body weight (B, n = 6-9, contains male mice), splenic weight as a fraction of total body weight (C, n = 6-9, contains male mice), KSL cells as a fraction of total splenic CD45$^+$ cells (E, n = 6-9, contains male mice), KSL cells as a fraction of total bone marrow CD45$^+$ cells (F, n = 6-9, contains male mice).

(D – E) In day 12 post-partum LIFR^{flox} mice with PDGFRα-Cre^{+} or PDGFRα-Cre^{-} littermates, body weight (D, n = 6-9, contains male mice), splenic weight as a fraction of total body weight (E, n = 6-9, contains male mice).

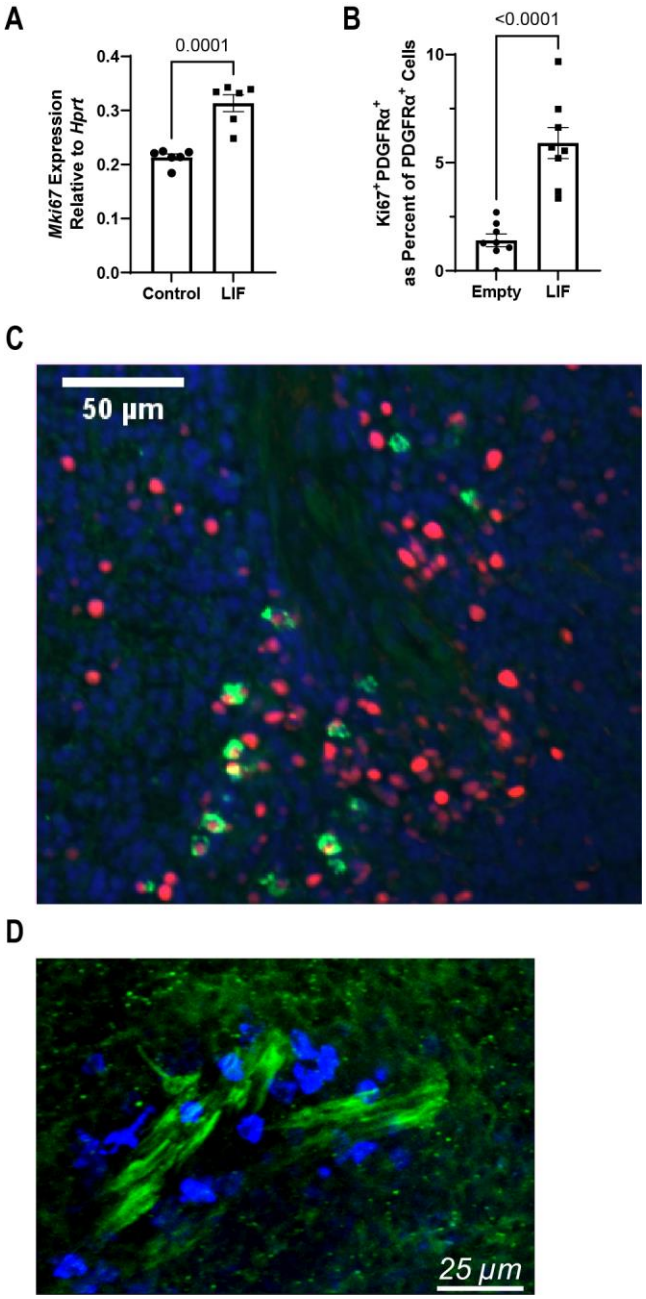

Figure 2.20: LIF directly expands the splenic niche by inducing niche cell proliferation

(A) Representative RT-qPCR expression data of *Mki67* from splenic ABS cells treated for 72 hours with 20ng/mL LIF (n = 6). (B) Fraction of splenic PDGFRα+ cell that are Ki67+ by immunofluorescence with 7 days of LIF overexpression or empty vector lentivirus control (n = 8). (C) Representative immunofluorescence image of the speen after LIF overexpression with PDGFRa^{+} cells in green, Ki67^{+} nuclei in red, and DAPI^{+} nuclei in blue. (D) Representative confocal image of spleen after LIF overexpression with PDGFRa^{+} cells in green and c-Kit^{+} cells in blue.

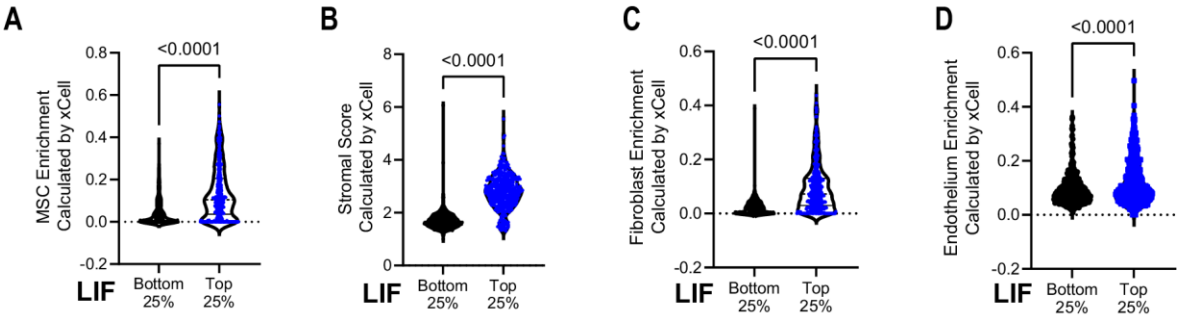

Figure 2.21: LIF also acts on locally to expand stromal cells more than endothelium

(A – D) Enrichment of MSCs (A), stromal scoring (B), fibroblasts (C), and endothelial cells (D) as calculated by xCell from RNA-seq data of human tumors split by top and bottom quartile of LIF expression (n = 416-417).

2.5.6 IL-1α and LIF are co-expressed in human cancers and have a cooperative myelopoietic response in mice

Re-examination of human tumor RNA-sequencing datasets identified that human breast cancer does not have the same cytokine profile as the murine PyMT model with respect to IL-1α, LIF, and G-CSF (Fig. 2.22A). However, human colon cancer does (Fig. 2.22B). Interestingly, human

pancreatic, stomach, brain, and bile duct cancer all have overexpression of both IL-1α and LIF relative to normal tissue while having only minor changes in G-CSF (Fig. 2.22C-F). These data suggest that the co-occurrence of IL-1α and LIF is potentially clinically relevant for a diverse set of human cancers. Due to their independent mechanisms in activating the splenic niche, we hypothesized that the interaction of these cytokines would increase myelopoietic output. To test this, we first injected mice with lentiviral constructs that were either empty or expressed LIF, followed by IL-1α. Mice that had previously received LIF had increased peripheral PMNs and splenic HSPCs and GMPs upon IL-1α injection compared to empty vector controls (Fig. 2.23A-C). This data suggests that LIF exerts a functional impact on hematopoietic capacity that can potentiate the myelopoietic impact of IL-1α. When combined with the identification of IL-1α and LIF overexpression in multiple human cancers, this illuminates a novel, potential mechanism by which cancers may generate a myeloid-biased immune environment by initiating EMH within the spleen (Fig. 2.23D).

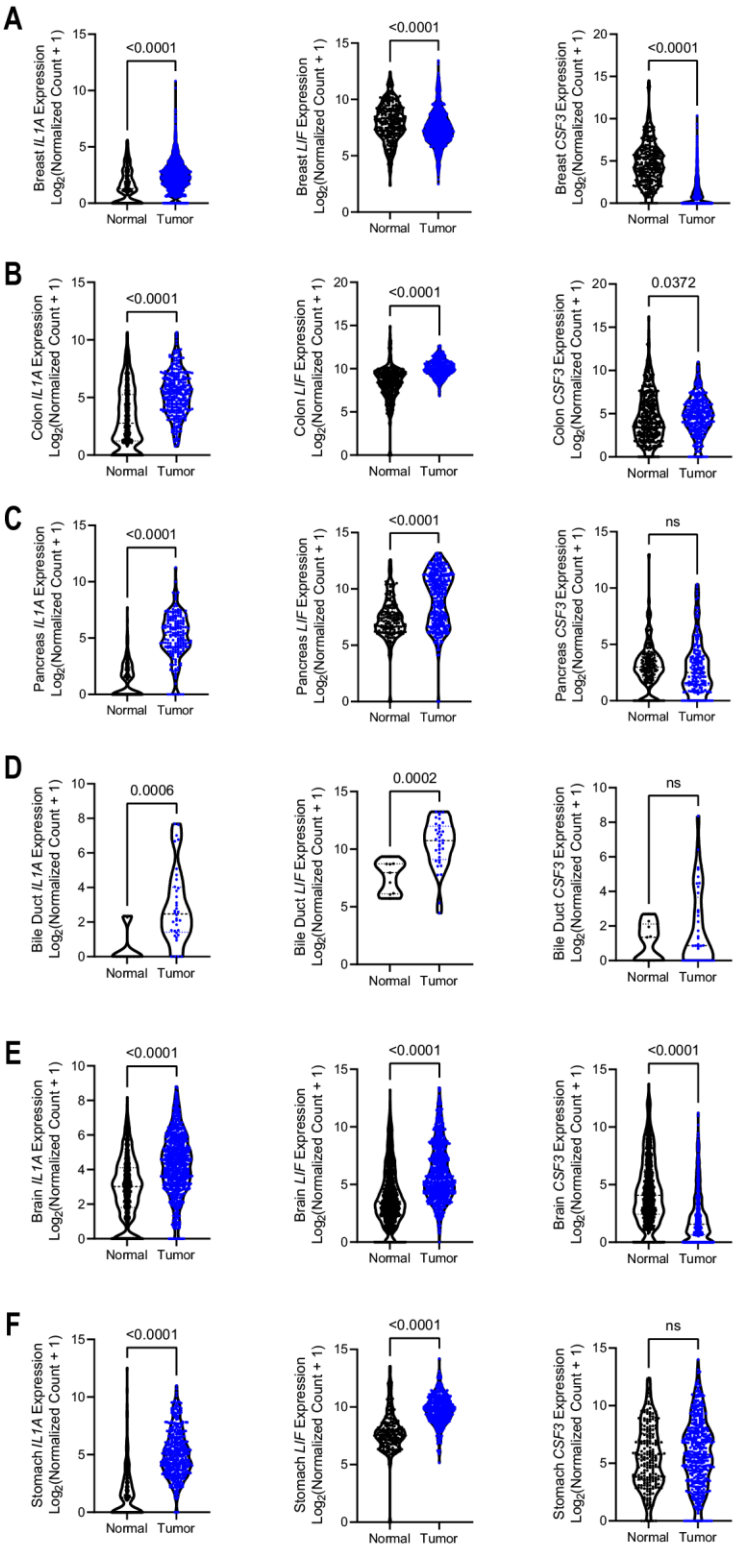

Figure 2.22: IL-1A and LIF co-expression is common in human tumor types

(A – F) RNA-seq expression of *IL1A*, *LIF*, and *CSF3* expression in tumor compared to normal tissue for breast (A, 292-1099), colonic (B, n = 288-349), and pancreatic (C, n = 171-178), bile duct (D, n = 9-36), brain (E, n = 689-1146), and gastric (F, n = 210-414) tumors.

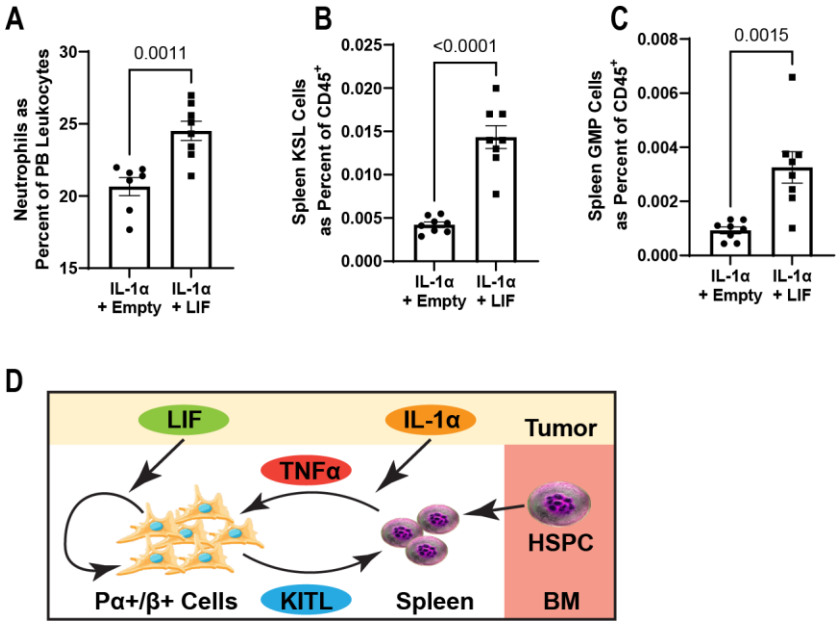

Figure 2.23: Human tumors co-express LIF and IL-1α which synergize in mouse models to potentiate EMH

(A – C) In mice with 10 days of LIF overexpression lentivirus or empty vector control lentivirus and 24 hours after treatment with 200ng IL-1α i.v., PMNs in the PB as a percent of total leukocytes (A, n = 7-8), KSL cells as a fraction of total splenic $CD45^+$ cells (B, n = 7-8), GMP cells as a fraction of total splenic $CD45^+$ cells (C, n = 7-8). (D) Our proposed model of parallel mechanisms for tumor induced EMH mediated by indirect inflammatory changes to HSPCs through IL-1α and direct proliferative effects on splenic ABS cells through LIF.

2.6 Discussion

Extramedullary hematopoiesis (EMH) can be viewed as a process undertaken to meet the immense demand for myeloid cells during pathology that exceeds the capacity for existing bone marrow progenitors. While EMH has been shown in a wide range of human inflammatory conditions and diseases (*138, 244, 246*), the mechanisms regulating EMH have not been clearly elucidated. Here, we show the spleen as a critical site of EMH during solid tumor pathology that drives increases in peripheral blood neutrophilia. Consistent with this is recasting the spleen as a primary lymphoid organ involved in sensing systemic inflammation and activating by expanding total hematopoietic capacity, often with a myeloid bias. This framing of splenic function is concordant with data demonstrating an origin for myeloid cells within the spleen during various inflammatory and non-inflammatory pathologic states in both humans and mice (*172, 248*). Previous work in a hepatocellular carcinoma mouse model has demonstrated that the absence of the spleen is sufficient to positively impact immune checkpoint blockade therapy (*247*). Additional work has suggested that absence of the spleen leads to significantly fewer tumors developing in an inducible model of lung cancer (*248*). We also show the reduction in the magnitude of tumor-induced neutrophil bias in the periphery following splenectomy. Interestingly, our paper finds less profound alterations to the bone marrow compartment when compared to other investigations of solid tumor induced effects on hematopoiesis. In particular, when compared to Casbon et al. 2015, although we find a trend towards increased BM cellularity and progenitor numbers, our results are less dramatic than their findings (*241*). Potentially, these discrepancies are the result of several differences between our experimentation and theirs including mouse genotypes, a genetic versus a cell line tumor model, and a longer timeframe in the genetic models. Additionally, we also speculate that increases to BM HSPCs seen in the

previously mentioned paper and others may contribute to increased splenic HSPCs through migration as observed in our model. These subtle differences and their impact on stem cell phenotypes highlight the nuance and limited understanding still present in the field of hematopoietic modulation by solid tumors.

While tumor manipulation of local immune cells within the tumor microenvironment has received significant attention, how tumor cells manage the immune system distally is less well-understood. In this paper, we demonstrate that profound expansion of hematopoiesis into the spleen occurs with breast cancer. We then identify two cytokines produced by tumor cells that have distinct but overlapping interactions with splenic HSPCs and stromal cells to expand the size and functional capacity of the splenic niche to accommodate increased myelopoiesis. We present novel findings that support this conclusion. First, splenic HSPCs accompanying tumor presence express a gene profile characterized by TNFα. Second, IL-1α released by the tumor cells acts distally to induce TNFα expression in HSPCs. Third, tumors indirectly activate splenic niche capacity in PDGFRα+/β+ stromal cells through local TNFα produced by inflammatory HSPCs. Fourth, tumors directly expand the splenic niche through LIF by inducing proliferation in splenic PDGFRα+/β+ stromal cell populations. Moreover, LIF receptor deletion in PDGFRα+ cells significantly reduced hematopoietic niche capacity within the spleen. These data extend the role of this underappreciated cell type by centering it as the activatable niche cell within the spleen. Importantly, identifying LIF as expanding this cell type adds to our appreciation of stromal cells as active members of inflammatory pathology. Our analysis of human tumor data hints that LIF may also have local effects supporting cancer-associated fibroblasts, a cell-type which has recently drawn attention as key member of the tumor immune environment (*285*).

Finally, the parallel mechanisms of IL-1α and LIF present in our model and in some human cancers can synergize to increase PMN production (Figure 7G).

Our data add depth and scope to the mechanisms by which cancers manipulate the host to generate a favorable immune environment for their growth, stretching as far up the differentiation hierarchy as primitive hematopoietic stem cells and their associated niche. One avenue that our paper focuses on is the cytokine axis established by tumor cells themselves. This focus uses an emerging classification of tumors by their functional effects that helps overcome heterogeneity both within and between tumor types and also makes comparisons of tumor pathology more congruous across species boundaries (*286*). Studying tumor-derived cytokines also dovetails with the recent developments in understanding the reaction of HSPCs to inflammation (*250, 287-290*). Our data also adds to the growing evidence supporting an active role in pathology played by HSPCs through inflammatory cytokine production (*247, 291*). Many cases of cytokine-induced changes to HSPCs result in myeloid lineage bias. This shift towards the production of myeloid cells benefits tumor growth while tending to harm cancer patients. Across multiple tumor types, including breast, colon, pancreatic, and gastric cancer, as well as a systematic review of all cancer types, a high neutrophil-to-lymphocyte ratio is an independent prognostic factor for survival (*254-257*). In addition to increased quantity, myeloid cells produced in communication with cancer cells have unique qualities that help drive cancer pathology, such as myeloid-derived suppressor cells (*247*). Our data expand the function of inflammatory cytokines produced by HSPCs beyond myeloid lineage biasing. Particularly, we provide data showing that TNFα produced by HSPCs can regulate the function of their own niche. In concert with tumor-produced LIF that expands the quantity of splenic HSPC niche cells, tumor-derived IL-1α induces TNFα production by HSPCs to alter niche function into

favoring increased EMH. This data warrants future studies addressing whether disruption of the local IL-1α/TNFα axis can impede EMH and how cell products of EMH induced by IL-1α and LIF impact the tumor microenvironment and cancer outcomes.

Chapter 3: Conclusion and future directions

Hematopoiesis touches every facet of vertebrate biology. Yet, a complete scientific understanding of it is still developing. Many open questions still remain in understanding how hematopoiesis develops into its adult form, the nature of self-renewal and differentiation, to what types of injury does it respond, how these responses occur and their impacts, whether these responses leave a permanent impact on the process, and through what effects can hematopoiesis transform into malignancy. These questions focus specifically on hematopoiesis itself and do not begin to address the impact of hematopoiesis on organ or organismal biology, let alone disease processes such as cancers of other organ systems. Still, as scientific techniques continue to advance, the questions that are able to be answered grows and further questions become uncovered while old questions become set for reexamination.

As the immune system begins to be appreciated as a major contributor to nearly every pathologic condition, hematopoiesis begins to be appreciated alongside it as one contributor to the phenotypic outcome of the blood and immune cells involved. As myeloid cells are known to be an important facet of pathologies whose functional contribution can vary based on the immune environment, the production of myeloid cells and their phenotype has been a focus of study for hematopoiesis during pathology. Since cancer is a major killer of people across the world (*225*) and is a pathology where myeloid cells play a central role in response to emerging immune directed treatments (*292, 293*), it is therefore sensible for studies of myelopoiesis to occur. While many potential avenues exist for additional therapeutics targeting aberrant myelopoiesis in the context of solid tumor pathology, investigations into the hematopoietic niche were a novel angle.

As with stem cell systems across the body, stem cells of the hematopoietic system are accompanied by a microanatomical environment known as the stem cell niche that supports and directs the function of the hematopoietic stem cells (*45, 258, 259*). While studies of the niche are even further in their infancy than studies of hematopoiesis more broadly, nonetheless information about the niche is nearing the cusp of being useful therapeutically. Additionally, in recent years, the ability of the hematopoietic system to expand outside of its quiescent niche within the bone marrow and conduct hematopoiesis at distal sites has become to be appreciated as a common response to pathology in both mice and humans (*136, 138, 242, 244, 246, 294*). Finally, stromal cells of the body are beginning to be understood as playing an important in immune responses more generally (*295-297*). These facts combined beg the question of how the stromal cells that comprise the hematopoietic niche of extramedullary sites respond to the inflammation generated during the presence of solid tumors and how those changes impact hematopoiesis at these sites.

In this body of work, my colleagues and I have identified a population of stromal cells in the red pulp of the spleen that is analogous to the stromal population of bone marrow that performs vital hemtatopoietic niche functions. Additionally, these cells are arrayed closely with hematopoietic stem and progenitor cells during extramedullary hematopoiesis. Finally, these cells respond to inflammatory cytokines produced as a result of tumor presence by expanding their capacity to support local hematopoiesis.

In the future, my work could be improved upon technically in several ways. First, though my work focuses on the spleen, other important secondary hematopoietic organs like the liver were not studied in detail. The liver is an interesting possibility because it is often cited as a companion to the spleen as sites of extramedullary hematopoiesis though it differs dramatically in tissue organization from the spleen. In particular, it has a paucity of fibroblastic stromal cells when

compared to the spleen and the bone marrow. In contrast, endothelial cells are also a potential stromal niche cell that are highly numerous in the liver and have been implicated as the niche forming cell (*147*). That all being said, the liver is well-understood to increase its hematopoietic capacity in response to cytokines like G-CSF and therefore it may similarly respond to cytokines like IL-1α and LIF. Together, this makes the liver an attractive target for expanding the number of organs capable of responding to cytokine stimulated extramedullary hematopoiesis. Though, just as there were technical hurdles that needed to be surmounted to study extramedullary hematopoiesis in the spleen, other hurdles would need to be addressed in the liver, some of which may be unsolvable. In particular, unlike the spleen, the liver is a vital organ and therefore testing its contribution to myelopoiesis would be daunting. Additionally, the liver has an immune cell compartment that is smaller and more difficult to access compared to the spleen.

Second, though my work did expand the tumor models investigated to include a lung carcinoma and a sarcoma, these did not fully capture the diversity of models present within tumor biology and the diversity of experimental conditions that could influence extramedullary hematopoiesis as a phenotype. Initially, one issue that is of interest is whether the location of the tumors, either *in situ* or in a heterologous location, impacts the development of extramedullary hematopoiesis. Our model system used heterotopic injections for cell line based tumors. However, PyMT-B6 and LLC carcinomas both have a primary location that could modulate the impact of tumor presence on EMH. This presents an experimental set-up that might yield interesting information when the effect on extramedullary hematopoiesis of identical tumors is compared between implantation in the orthotopic site or the heterotopic site. This would isolate the stromal environments impact which has been shown to be important for other aspects of cancer biology. Additionally, genetic or inducible models could be further explored that have the added benefits

of being potentially better models of human disease and of occurring over a longer time period. Potentially, these changes to the tumor model would also alter the development of extramedullary hematopoiesis in the spleen or other organs and ideally make findings more relevant to human disease.

Third, in response to reviewers' comments, confocal imaging was performed on the spleen in the context of LIF expanded hematopoiesis. This technical modality offered a unique insight into the splenic niche that was not fully utilized in my paper. Specifically, the shape of stromal cells is not conducive to easy study by flow cytometry or thin section immunofluorescence. Because these cells tend to have large, irregular shapes and intimate connection with the extracellular matrix, they are difficult isolate for single cell suspension directly out of the spleen. Given the proper time to set-up the technique more thoroughly, aspects of the niche such as the spatial relationship of the hematopoietic progenitors and the stromal cells could have been assessed in complementary ways to the existing data. Also, this technique could have been used to validate single cell RNA-seq data and the *in vitro* data regarding aspects of the stromal cells. In particular, it would have been beneficial to see the co-expression of the PDGFRα and PDGFRß markers on cells in the spleen and to see expression of KIT ligand or other niche associated factors. With a basic understanding of the 3-dimensional layout of the splenic niche, quantitative experiments could be designed such as enumeration of the stromal niche cells or of hematopoietic/niche clusters with or without tumor. This additional experimental readout would bolster claims made using other techniques and complement the inherently spatial organization of the hematopoietic niche.

Fourth, though single cell RNA-sequencing was successful at capturing changes to the hematopoietic precursor compartment within the bone marrow and spleen following tumor transplantation, it did not capture much information regarding changes to the stroma. Specifically,

though a small number of stromal cells were captured, they did not match our expectation for the number or diversity of stromal cells within either organ. Potential causes of this technical issue are numerous, and our current information on how this occurred is incomplete. However, existence of papers which successfully profile stromal cells in the bone marrow and other locations exist. Therefore, we can largely rule out a technical incompatibility existing between the sequencing modality and the cell types. However, though they are able to be sequenced, that does not mean that whole cell single sequencing is the ideal platform. For instance, neurons, another cell type with complicated morphologies, are not well samples by whole cell sequencing and have more successfully been captured by single nucleus RNA-sequencing (*298*). This may be especially true when stromal cells directly compete for capture with hematopoietic cells that are generally excellently sequenced by whole cell modalities. It is possible that changes to analysis during flow cytometry could improve capture of stromal cells by, for instance, expanding the singlet and SSC/FSC gates to allow for larger and more irregular cells. However, perhaps following technical innovation in the neuronal field such as single nuclear sequencing would provide better capture. This would be helped by novel genetic labeling markers specifically designed for nuclear capture and flow cytometry, such as the NuTRAP mouse (*299*). Once the technical barriers to capture stromal cells using single cell RNA-sequencing is overcome, this opens the possibility for comparisons of the gene expression between stromal populations in the bone marrow and spleen with or without tumor presence. This data could shed further light on the genetic mechanisms that are allowing for the expansion of hematopoiesis and enhanced myelopoiesis within our tumor models.

Finally, certain experiments that would have answered interesting questions about the stromal niche cells were hindered by poor reagents. In particular, I found antibodies against surface

markers for stromal cells to be very lacking. Though I was able to get useful data from antibodies against surface KIT ligand, this was polyclonal antibody with high background. The development of a monoclonal antibody against this target would have expanded my experimental capabilities, particularly from the perspective of imaging. Additionally, there were several surface proteins that were identified as being marker genes for these stromal niche cells that no antibodies whatsoever. In contrast to studying hematopoietic cells, I was surprised by the dearth of commercial, functional antibodies against stromal antigens.

Though there are areas where experimentation is possible to further solidify my findings, our current data still presents a powerful and useful picture from which one could build. With my data as a foundation, we can begin to speculate about the further areas of exploration that follow from both my research and from existing pieces of literature and public data.

An obvious next step to bridge the translational impact of my work would be to use human cells and tissue samples to begin to understand the potential relevance of my work to clinical medicine. Human mesenchymal stem cells are phenotypically very similar to the splenic stromal cells I identified in my work (*107*). Importantly, they have been demonstrated to possess the capacity to maintain human hematopoietic stem cells *in vitro (107)*. This begs the question whether the effects we have seen with mouse splenic stromal cells would be replicated by these human mesenchymal stem cells as well. As TNFα has broadly distributed receptors, it is reasonable to assume that these cells do respond to this cytokine. However, the CXCR2 ligands of human differ slightly from mice and includes an important cytokine IL-8/CXCL8 (*300*). It is possible that the chemokines released by MSCs in response to inflammatory cytokines like TNFα might not match the mouse data. Additionally, CD34+ human hematopoietic progenitors are readily available, and it would be relatively trivial to assess whether these cells respond to IL-1α similarly to their mouse

counter parts. As for being able to study organismal physiology in the context of the solid tumors, the acquisition of samples is a clear hindrance in this case. In particular, spleens are rarely biopsied while the patient is alive or without a secondary injury and therefore studies of hematopoiesis within this context would prove difficult. However, post-mortem samples may provide an interesting source of samples though they would be limited to histologic studies. Additionally, liver biopsies are more commonly performed and may offer insight into extramedullary hematopoiesis, though at a different site than the spleen.

While this work focuses on the expansion of myeloid-biased hematopoietic capacity in the spleen, the potential exists for there to be conditions in which lymphoid biased hematopoiesis occurs at extramedullary sites. One disease state that seems plausible for the presence of lymphoid biased hematopoiesis if it exists are during viral infection. In particular, Epstein-Barr virus is well-known to cause splenomegaly which throughout the course of my work has been a strong indicator of ongoing splenic hematopoiesis (*301*). However, unlike solid tumors, the predominant phenotype in the peripheral blood is not neutrophilia but instead is lymphocytosis (*301*). Additionally, there is a potential reasoning for the extra production of lymphocytes during viral infection because they can produce antibody to neutralize the virus through B-cell production and recognize virally infected cells through CD8+ T-cells. B-cells in particular would likely be the target of enhanced lymphopoiesis as the germinal center reaction leads to large scale B-cell death and may need additional input from progenitor cells to maintain the reaction (*302*). The lymphocytic response from Epstein-Barr virus also contains gamma-delta T-cells and NK cells that may also be a target of enhanced lymphopoiesis because they do not require as in-depth educational steps during development compared to B- or T-cells but are still important aspects of the immune response (*301*). Additionally, enhanced lymphopoiesis could be a mechanism by which non-self-tolerant T-

cell clones are produced if for instance the mechanisms of increased T-cell generation leads to decreased selective pressure and clones surviving that would normally be deleted by tolerance mechanisms in the thymus. Despite this logic for the existence of enhanced lymphopoiesis, evidence for its existence is sparse. One tantalizing article describes overexpression of IL-21 as being able to dramatically increase the number of Lin-/c-Kit+/Sca-1+ cells within the spleen (*303*). Unlike many cytokines implicated in myeloid-biased hematopoiesis, the function of IL-21 has generally been described in relation to germinal center reactions and its role in modulating B-cells (*304*). This opens the possibility that it could be acting on stem or progenitor cells to increase B-cell production for use in the germinal center reaction. This indicates that just as there exist cytokines like G-CSF, which act on mature myeloid cells as well as having an effect of hematopoietic progenitors, there are cytokines, while whose predominant function is on mature lymphocytes, that have effects also on the progenitor level. Together, I think that there are compelling first principle reasons to search for evidence of enhanced lymphopoiesis in the context of pathology. However, there are also compelling reasons to believe that the scope of pathologies that have this property and that the degree to which it is enacted are more limited compared to emergency myelopoiesis.

Further extramedullary sites in mice and humans are deserving of study outside of the spleen. In the context of solid tumors, many organs and locations were identified as containing cases of extramedullary hematopoiesis (*136, 138*). While sites that would be expected were identified such as the spleen and liver, several unexpected places were also found such as the paraspinal region, peritoneum, bronchia, adrenal gland, endometrium, pancreas, and ureter (*135, 136, 138*). The breadth of the sites capable of conducting hematopoiesis is astonishing and hints at a broader relevance of this phenomenon than is commonly appreciated. Additionally, as has

been discussed in the literature, extramedullary hematopoiesis is likely to be underreported for a couple of reasons (*294*). First, extramedullary hematopoiesis does not produce distinguishing features on imaging and requires biopsy to confirm, which may not be desirable in many cases. Second, clinicians are not often aware of the possibility for hematopoiesis to occur outside of the bone marrow and may not be looking for it to occur when treating patients. That being said, of the sites of extramedullary hematopoiesis, two in particular standout to me: the skin and lymph nodes.

The skin is of interest for study because cutaneous hematopoiesis, the 'blueberry muffin rash', is a hallmark of pre-natal TORCH infections (*137*). Cutaneous hematopoiesis is odd because unlike extramedullary hematopoiesis in the spleen or liver, it is never a predominant site of developmental hematopoiesis. Indeed, the skin would seem at first glance to be an inadequate site for hematopoiesis because it does not offer the security of the bone marrow nor the vascular access of the spleen or liver. Perhaps its appearance in response to overwhelming pre-natal infection belies drastically expanded hematopoiesis in other sites throughout the body that is still unable to match the scope of the infection.

Lymph node hematopoiesis is of interest because evidence suggests they form the predominant site of extramedullary hematopoiesis in the presence of solid tumors. Conceptually, lymph nodes and spleen share a function as organs of immune surveillance for different fluid compartments in the body, the lymph and blood, respectively. Additionally, on a finer level, the microanatomical organization of the two organs also rhymes. However, one key difference in the context of solid tumors is that the lymph node will be experiencing an increased load of cytokines from the tumor mass. Assuming that the lymph node stroma is as equally reactive as the spleen, one would expect that lymph node hematopoiesis would be more dramatic than in the spleen itself.

However, draining lymph nodes are also common sites of first metastasis and therefore tumor growth in the lymph node may obscure any ongoing hematopoiesis if it is occurring.

The diversity but also the hierarchical nature of locations for extramedullary hematopoiesis indicates that not all stromal cells that form the niche are equally functional or potentially require additional signals to activate. One potential line of investigation that is opened due to my work is to profile the tissue specific differences between PDGFRα/ß cells across tissues to understand how their niche functions may be regulated by the tissue environment. Current literature suggests that mesenchymal stem cells across tissue types do have important phenotypic differences (*149, 305*). Comparative studies across organs may lead us to a better understanding of what drives niche activity and why it does predominantly occur in only a few organs and only have a large permanent activity in the bone marrow. One location that has many cells with this phenotype but does not appear to have a large capacity for extramedullary hematopoiesis is muscle tissue (*267*). Potentially, there is a specific differentiation or genetic profile required to active niche potential in these cells that is not present in the muscle.

Following from this idea is the nature of these splenic stromal cells themselves and whether they possess stemness of their own. One of the defining features of mesenchymal stem cells is their stemness (*112, 306*). For instance, bone marrow mesenchymal stem cells are consistently reported as being capable of differentiating into osteoblasts, chondrocytes, and adipocytes (*112, 306*). However, as central as this characteristic may appear, there is debate about the degree of stemness in these cells. Returning to the example of the bone marrow mesenchymal stem cell, those cell types that are reported to be downstream of the stem cells themselves are all normal components of the bone stromal environment. In contrast, none of those cell types are reported under homeostasis within the splenic environment. Furthermore, mesenchymal stem cells from the

skeletal muscle possess myogenic capabilities (*307*). Potentially stemness of mesenchymal stem cells and the breadth of their differentiation potential is also tissue dependent along with their niche potential.

Another set of open questions under the heading of mesenchymal stem cell identity is their *in vivo* identity and specifically their association with vasculature (*308*). Several publications have identified mesenchymal stem cells as having a close spatial relationship with vasculature (*308*). Some have even claimed that pericytes are the true origin of mesenchymal stem cells (*308*). However, pericytes are identified as being PDGFRß single positive as compared to mesenchymal stem cells as having expression of both PDGFRα and PDGFRß (*309*). Whether this represents a true distinction of cell type or a change to the culture environment is an interesting question. One alternative question of relevance to cellular therapy modalities involving mesenchymal stem cells is what is the identity of these cells after injection. Potentially splenic mesenchymal stem cells may offer a useful experimental system for studying this. For instance, it would be reasonable to assume that cells with minimal mobility such as mesenchymal stem cells would be caught within the spleen when injected systemically. Studies of the fate of these cells in the spleen may shed light onto the identity of mesenchymal stem cells. Furthermore, if mesenchymal stem cells are able to successfully colonize the spleen after injection, experiments involving genetic manipulation of these cells could be a useful tool for studying extramedullary hematopoiesis. One could imagine removing a potentially critical factor from these injected cells and then investigating whether their niche function is preserved. Either way, this close stromal/endothelial relationship may be informative when deciphering the existing literature and designing future experiments. Many studies identify endothelial cells as contributing to the hematopoietic niche. However, care must

be taken in interpreting these data because it is not always straightforward to fully dissociate these two cell types.

While my experiments have focused on the myelopoietic of the splenic stromal cells, there exists evidence to suspect that they also possess lymphopoietic activity as well. Most importantly these cells according to ImmGen and single cell RNA-sequencing of the bone marrow are the strongest producers of IL-7, a factor required for lymphopoiesis, and a strong producer of Flt3 ligand, another lymphopoeitic factor. This may be relevant to the exploration of enhanced lymphopoiesis discussed earlier. One could imagine a chain of events similar to those described in my work leading to expanded lymphopoesis instead of myelopoiesis. Of particular interest is the ability of activated T-cells to express LIF (*310*). There than exists a direct circuit from activated T-cells in the spleen releasing LIF onto splenic stromal cells and activating them for increased hematopoiesis. With the proper cytokine milieu, this could directly stimulate the production of more T-cells to continue the immune response. Taken from another angle, this T-cell/mesenchymal stem cell interaction may also be important in the thymus where T-cell progenitors are directly produced and thymic mesenchymal stem cells are present.

On the flip side of the coin from what factors mesenchymal stem cells produce is what factors mesenchymal stem cells can respond to. In particular two factors related to our study might be of interest to future studies: oncostatin M and IL-1 family members.

Oncostatin M is an IL-6 family cytokine but more importantly it also can signal through the LIF receptor and its own private oncostatin M receptor (*311*). Like we have shown for its family member LIF, oncostatin M has activities on the hematopoietic system such as driving myelopoiesis (*312*). Oncostatin M also has a well-recognized effects on stromal cells (*310*).

Oncostatin M was also used a part of the cell culture media for human mesenchymal stem cells in a paper where they demonstrated that these cells have niche capability (*106*). Additionally, oncostatin M receptor is expressed by PDGFRα/ß cells both by ImmGen and single cell RNA-sequencing. However, oncostatin M differs from LIF in important ways. Most importantly, oncostatin M can signal through STAT1 in addition to STAT3 (*311*). This changes what genes oncostatin M activates. Additionally, oncostatin M has different cellular sources than LIF. Though both LIF and oncostatin M are expressed by mast cells in our single cell dataset of the spleen, oncostatin M expression is also reported across numerous immune cell subset including T-cells, monocytes, macrophages, and neutrophils (*311*). Given the breadth of cell types and increased representation of immune cell types, the disease that oncostatin M has been recognized in are distinct from LIF. In particular, oncostatin M has been widely associated with joint disease. Importantly, oncostatin M is found in the synovial fluid of patients with rheumatoid arthritis (*313-315*). Additionally, oncostatin M was recently identified as being a biomarker of failure to respond to anti-TNFa therapy in inflammatory bowel disease (*316*). Together, these data indicate that oncostatin M is an important cytokine associated with pathology and expanded hematopoiesis that has overlapping yet distinct effects compared with LIF.

Another receptor for a stromal active cytokine that is expressed on PDGFRα/ß cells that might have broader implications for pathology is IL-1 receptor. In addition to its expression on hematopoietic stem cells, IL-1 receptor also has strong expression on PDGFRα/ß cells. Indeed a couple of papers have identified the importance of stromal cells in responding to IL-1 inflammation (*317, 318*). However, the effects of ligands to this receptor on these cells is poorly understood. Additionally, IL-1 family has two cytokines with different expression patterns and biology: IL-1α and IL-1ß (*173*). In model of Kawasaki disease vasculitis, both IL-1α and IL-1ß

were both required in the hematopoietic compartment while IL-1 receptor was required in the stromal compartment for disease (*317*). In another paper, IL-1 receptor signaling in intestinal stroma was identified as a being an important driver of neutrophil recruitment (*318*). Together, both oncostatin M and IL-1 represent interesting avenues to continue studying the response of stromal niche cell to systemic inflammation during disease.

Another aspect of stromal response to cytokines that begs to be addressed is the necessity of LIF receptor expression in stromal cells. Though we were able to identify hematopoietic defects in mice with conditional deletion of LIF receptor in PDGFRα+ cells, we not able to identify a mode of death for these animals. However, several potential mechanisms are possible. Given the well-established role of LIF in stimulating growth of stromal cells, we hypothesize that death in these animals occurs where there is a need for cells that is required for life. In particular, we believe the intestinal tract and neural system present the most likely mechanisms. Mice lacking PDGFRα develop abnormal intestinal tracts characterized by the loss of pericryptal mesenchymal cells and misshapen villi (*319*). Additionally, these PDGFRα cells make up the intestinal epithelial stem cell niche and when critical stemness factors are deleted from this population, epithelial response to injury is negatively affected (*320*). These data suggest that the population of PDGFRα expressing cells within the intestinal stroma play an important role in intestinal development and homeostasis. It is therefore reasonable to assume that disrupting the growth of these cells might lead to impaired nutrient uptake and anti-bacterial mucosal defenses that could lead to a failure to thrive phenotype akin to what we observe. On the other hand, oligodendrocyte precursor cells are an important cell type of the nervous system that is capable of generating new oligodendrocytes to myelinate neuron axons. These cells are well-recognized as being PDGFRa+ (*321*). Furthermore, LIF signaling has been identified as being able to induce proliferation within the oligodendrocyte precursor

population (*199*). Loss of this cell population may have stunted neuronal development necessary to cause the early death of these animals. Together, the striking phenotype of animals with LIF receptor signaling loss in PDGFRα+ cell highlights how these cells represent an important constituent of many organs and that LIF signaling is vital to their homeostasis.

Although our system did not require LIF receptor in the endothelial compartment, its high expression presents an interesting question as to its utility in this cell type. However, research on this topic presents a confusing picture. One study of LIF knockout animals found that LIF inhibited VEGF expression (*322*). Another study found that LIF inhibits bovine angiogenesis *in vitro* (*323*). Finally, LIF has been shown to lead to differentiation of cardiac stem cells into endothelium (*324*). In contrast, another study found that LIF preserves stemness in endothelial progenitor cells (*325*) and yet another paper found that LIF was mitogenic to choroidal endothelium within the bovine retina but inhibitory to aortic endothelium (*326*). Together these papers present a muddied picture of what the function of LIF receptor signaling might be to endothelium. However, perhaps further experimentation and use of modern techniques might be able to add clarity to this field. One potential avenue for exploration would be to see whether LIF helps endothelium from the bone marrow or spleen act as niche cells for hematopoietic progenitors in a co-culture system.

On the topic of co-cultures, our paper delineates a novel method for study of extramedullary hematopoiesis by co-culture bone marrow hematopoietic progenitors with splenic stromal cells. When grown together, this system is able to continually produce hematopoietic progenitors for at least 21 days. This is a remarkable system that can potentially offer further insight into the mechanisms of niche activation during extramedullary hematopoiesis while also still able to be improved technically. First, further characterization of the hematopoietic cells generated in co-culture could be undertaken. This might be potentially fruitful because the flow

cytometry plots of the Lin- compartment show an odd phenotype of cells compared to either the bone marrow or spleen. A more in-depth panel could illuminate what these cells are becoming. From IL-7 and Flt3 ligand expression data discussed earlier it seems likely that the majority of cells produced in this system are of lymphoid or dendritic cell lineages. Second, experiments could be conducted to see how cytokine perturbations to the co-culture system affect hematopoiesis. Cytokines that might be of particular interest include IL-1α, LIF, OSM, and IL-1ß. Readouts could include number and differentiation state of the hematopoietic cells derived from progenitors in co-culture. Third, genetic knockouts could be made to either component of the co-culture and the outcomes of hematopoiesis could be assessed. Of particular interest might be IL-7 expression in the mesenchymal stem cell fraction or IL-1 receptor expression in either fraction. Finally, this system is amenable to imaging modalities such as live-cell imaging. This could be used to interrogate with increased precision the inter-cellular signaling occurring between hematopoietic progenitors and their niche. With these tools, further understanding of the extramedullary hematopoietic niche could be acquired.

In contrast with some implications of my work on pathology-associated splenic hematopoiesis, the spleen is not completely hematologically quiescent in the mouse during homeostasis. While splenic erythropoiesis is understood to occur both in the liver and spleen during development and continue until week 7 post-partum in the spleen (*327*), the erythropoietic role of the spleen during adulthood is still being determined. Multiple papers have identified the spleen as an important site of stress erythropoiesis during pathology. For instance, the spleen in a mouse model of atherosclerosis has increased levels of erythroid progenitors in the spleen (*328*). In particular, multiple lines of evidence suggest that G-CSF can induce the shift of erythropoiesis from the bone marrow to the spleen (*153, 329*). Contrary to the assertions of these papers, some

of my data suggests that the mouse spleen conducts erythropoiesis at baseline. In particular, when scRNA-seq of splenic progenitors at homeostasis were analyzed alone, my single cell RNA-sequencing data suggests that most prominent c-Kit+ progenitors of the spleen at homeostasis are erythroid progenitors. Accompanying this, flow cytometry data from the homeostatic spleen does include a small population of Lin-/Sca-1-/c-Kit+/CD34-/CD16/32- cells consistent with megakaryocyte and erythrocyte progenitors. Together these data open the possibility for understanding the degree to which the spleen can conduct erythropoiesis at homeostasis as well as during disease.

The implications of baseline hematopoiesis in the spleen skewed towards erythropoiesis bring up another potential line of inquiry: the unique properties of tissue hematopoietic stem and progenitor cells. As has been discussed previously, more and more sites around the body are capable of conducting hematopoiesis given the correct pathologic or developmental state. Recent years have seen a growth in experimentation supporting the importance and unique characteristics of immune cells that take residence in different tissues. Taken together, this implies that hematopoietic stem cells may also have unique properties depending on their site of residence. In fact, a recent paper shows that there exists a reservoir of megakaryocyte progenitors within the lungs that contributes significantly to platelet biogenesis (*330*). One could even make the case that the thymus is also a site where tissue specific effects lead to the preferential production of T-cells. For instance, double negative thymocytes, the most primitive T-cell progenitor are known to have multipotency and possess a Lin-/c-Kit+/Sca-1+ surface phenotype (*331*). Looking further afield, the bursa of Fabricius, a primary lymphoid organ in avian animals, and the ileal Peyer's patch of ruminants are sites of B-cell specific hematopoiesis (*332*). When considered in the light of tissue specific hematopoeisis, my own data supports this by suggesting that the spleen may be a

permanent site of erythropoiesis that can be expanded during stressors. Taking cues from other immunologic fields, one is tempted to ask whether tissue resident hematopoietic progenitors of tissues like the spleen vary qualitatively from those that migrate there transiently and to ask what tissue specific signals push differentiation towards specific lineages. At the confluence of both solid tumor pathology and tissue specific hematopoiesis is the question of whether hematopoiesis can occur within tumors themselves. While outwardly slightly outlandish, a recent paper published on the identification of hematopoietic stem and progenitor cells within human glioblastomas (*333*). These observations reveal that we may be on the cusp of the developing field of tissue specific hematopoiesis.

A contrasting idea to tissue specific hematopoiesis is to ask why the bone marrow is the predominant site of homeostatic hematopoiesis in adults and why it does not respond as vigorously to stress as other sites such as the liver and spleen. One potential idea is that bone marrow is already filled with stromal progenitors tasked with producing bone and cartilage and thus has a large supply of potential niche cells to direct hematopoiesis. During stress and due to the inelasticity of the marrow space, this site cannot expand further to accommodate increased hematopoietic capacity. This restriction might make the two most prominent vascular organs of the abdominal cavity a natural site for expanded hematopoiesis.

One significant point of interest that my data does not address is the potential for different tumor types to impact extramedullary hematopoiesis or interact with stem cell niches in different ways. Some mechanisms might be more obvious like the potential for different hematopoietic active cytokines to be produced by different tumor types and have activity systemically. However, it is also of interest how different stages or capabilities of similar tumor types might interact with the hematopoietic system. For instance, one could imagine that metastases might have stronger

impacts on extramedullary hematopoiesis because their cytokine products might have better vascular access. Additionally, c-KIT is also known to be expressed by certain solid tumor types (*334, 335*). One fascinating aspect is that gastrointestinal stromal tumors are likely to harbor mutations in c-Kit. This is utilized in treatment strategies as c-Kit is targeted by the drug imatinib, and tumors generally have impressive responses to the treatment (*336*). This also hints at the potential for these cells to require KIT ligand. Taken further, it is possible that successful metastasis of these tumors requires the presence of KIT ligand expressing niche cells in the metastatic site to support growth. Speculating on the impact hematopoietic niche cells in solid tumors also opens the doors for speculating on the importance of hematopoietic niches for the growth of hematopoietic tumors (*337*). While the hematopoietic niche is certainly important for liquid tumors, a couple of second-order aspects interest me in particular: mechanisms of bone marrow failure and the potential for tissue specific niches to favor certain cancer types. One aspect of the stem cell niche is maintaining quiescent stem cells. One way that bone marrow failure has been hypothesized to occur with acute myeloid leukemia is through enforced quiescence of non-leukemia stem cells (*338*). This may be a result of signaling from the leukemic cells through the stem cell niche. Of the most likely places for metastasis, the spleen and lymph nodes were the most likely metastatic organ in acute lymphoblastic, chronic lymphocytic, acute myeloblastic, and chronic myelocytic leukemias (*339*). While later developmental stages must be taken into account, the presence of the lymph nodes and spleen as sites of metastasis may in part be due to their receptivity for hematopoietic progenitors. Interestingly, cancers such as acute lymphoblastic leukemia are accompanied by production of cytokines associated with extramedullary hematopoiesis in the spleen such as TNFα and IL-1ß (*340*).

Accompanying the idea of developed leukemia interacting with the stem cell niche is the potential for the development of leukemias. Therapy-related myeloid leukemia are class of myelodysplastic or acute myeloid leukemia that occur as a complication to cytotoxic therapy given for other diseases (*341*). While hematopoietic malignancies are highly likely to develop therapy-related leukemia, non-hematopoietic tumors such as breast cancer also develop therapy-related leukemias. One study identified that the presence of G-CSF in therapeutic modalities increased the risk for developing therapy-related leukemias even when controlled for other chemotherapeutics (*342*). This potentially indicates that the expansion of hematopoiesis by G-CSF may provide increased selective pressure for the development of pre-leukemic clones by increasing the pool of clones before it is reduced by chemotherapeutics. Additionally, different sites of hematopoiesis may lead to the development of leukemias than would be assumed by differentiation. For instance, one paper reports that the development of T-cell acute lymphocytic leukemia is dependent not on the thymus but the spleen (*343*). Together, these data indicate that extramedullary stem cell niches and the process of expanding hematopoiesis potentially play important roles in leukemogenesis.

The results of my graduate work demonstrate interesting biology regarding extramedullary stem cell niches and cancer. However, as is hopefully demonstrated in the above discussion, these results, when interpreted in light of existing scientific knowledge, offer increased opportunities to understand biologic questions plaguing a diverse array of disease relevant fields.

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