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Differential Recruitment of Bone Marrow-Derived Cells Into the Injured Retina

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DIFFERENTIAL RECRUITMENT OF BONE MARROW-DERIVED CELLS INTO THE INJURED RETINA

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A DISSERTATION

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ABSTRACT

The hematopoietic system, which resides in the bone marrow in adults, maintains the constant turnover of blood cells in the circulatory system and supplies inflammatory/vascular reparative cells to the retina. Chronic metabolic diseases disrupt hematopoietic homeostasis, suppressing the generation of bone marrow-derived reparative cells in favor of pro-inflammatory cells. While much research has been done on development and disease-associated changes in hematopoiesis in the long bones and how mobilization to the retina is affected, the calvarium's hematopoiesis is underexplored. This dissertation investigated hematopoiesis in postnatal development and in disease, comparing calvarium hematopoiesis to that of long bones. The findings showed that hematopoietic cells are recruited into the retina prenatally, distributed over the entire retina at birth and are critical for retinal angiogenesis in development. In addition, the calvarial marrow showed delayed hematopoietic development, delayed reconstitution of blood but enhanced engraftment of hematopoietic stem and progenitor cells (HSPCs) compared to long bones. Using retina ischemia-reperfusion injury model, it was observed that the calvaria marrow contributes more neutrophils and myeloid angiogenic cells (MACs) to the retina than the long bones following acute injury, even though the proportion of neutrophils and MACs at baseline were not significantly different between the two compartments. In chronic type 2 diabetes, it was observed that the calvarial marrow showed a resistance to the damaging effects of type 2 diabetes. The calvarium underwent slower bone

deterioration, reduced buildup of fat content and less vascular degeneration compared to the tibia during chronic type 2 diabetes. This cumulatively resulted in a relative preservation of hematopoietic stem and progenitor cell function and an increase in erythroid lineage cells in the calvarial marrow and may explain, in part, why diabetic retinopathy is delayed in its development as the source of reparative cells for the retina comes from this protected compartment. Taken together, this dissertation has uncovered novel details about intramedullary hematopoiesis that shows that hematopoiesis in different bone marrow compartments is functionally different and important for the development of a healthy retina. It also highlights the significance of the calvaria bone marrow as a special source of reparative cells for the retina and survival of HSPCs and also a potential therapeutic target for delivery of therapeutic agents aimed at preventing or treating diabetic bone marrow damage and retinopathy.

Keywords: calvarial marrow, retinal angiogenesis, retinal ischemia-reperfusion, long bones, development of hematopoiesis, relative preservation of hematopoiesis

DEDICATION

This dissertation is dedicated to my mom, Mercy Kwaw, whose hard work has supported me throughout my academic life, and to the rest of my family to whom I will forever be grateful.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure *Page*

INTRODUCTION

DEVELOPMENTAL DELAY IN CALVARIAL HEMATOPOIESIS AND THE DIFFERENTIAL RECRUITMENT OF BONE MARROW-DERIVED CELLS INTO THE INJURED RETINA

LIST OF ABBREVIATIONS

AGM	aorta-gonad-mesonephros
BMD	bone mineral density
CACs	circulating angiogenic cells
CAR	CXCL12-abundant reticular
C/EBPα	CCAAT/enhancer-binding protein alpha
CFU	colony forming unit
CGRP	calcitonin gene-related peptide
CMP	common myeloid progenitor
CSF	cerebrospinal fluid
CXCL12	C-X-C motif chemokine ligand 12
CXCR1	C-X-C motif chemokine receptor 1
CXCR4	C-X-C motif chemokine receptor 4
DAMPS	damage-associated molecular patterns
DMHCA	N, N-dimethyl-3β-hydroxy-cholenamide
ECFCs	endothelial colony forming cells
ECM	extracellular matrix
FACS	fluorescence-activated cell sorting
FBM	fetal bone marrow
FL	fetal liver

G-CSF	granulocyte colony-stimulating factor
GCL	ganglion cell layer
GFP	green fluorescent protein
GMP	granulocyte-macrophage progenitor
HPCs	hematopoietic progenitor cells
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
ICAMs	intercellular adhesion molecules
IL-3	interleukin-3
IL-6	interleukin-6
INL	inner nuclear layer
IPL	inner plexiform layer
LT-HSCS	long-term hematopoietic stem cells
LTRS	long-term reconstitution cells
LXR	Liver X receptor
MACs	myeloid angiogenic cells
M-CSF	macrophage colony-stimulating factor
MEP	megakaryocyte-erythroid progenitor
MMPS	metalloproteinases
MPPS	multipotent progenitors
NBL	neuroblast layer
ONL	outer nuclear layer
OPL	outer plexiform layer

PBS	phosphate-buffered saline
PGF	placental growth factor
PPARγ	peroxisome proliferator-activated receptor γ
RAGEs	receptors for advanced glycation end products
RAMP1	receptor activity modifying protein 1
SCF	stem cell factor
SDF-1	stromal cell-derived factor 1
ST-HSCS	short-term hematopoietic stem cells
VCAMs	vascular cell adhesion molecules
VEGF-A	vascular endothelial growth factor-A

INTRODUCTION

Hematopoiesis Overview

Hematopoiesis is the process by which all blood cells- erythrocytes, platelets, neutrophils, macrophages, monocytes, natural killer cells, T-lymphocytes and B-lymphocytes, are continuously produced throughout life [1, 2]. It is the expansion and subsequent differentiation of blood-fated stem cells in the bone marrow into these mature phenotypes that are released into circulation [3]. It has long been known that even in the healthy state, most blood cells have a rather short lifespan and undergo constant turnover (long-lived quiescent stem cells and memory T and B cells the exception), making the hematopoietic system one of the most dynamic in the body [4, 5]. Approximately one trillion blood cells are produced daily in humans, making blood the most regenerative tissue in the body[6]. Although early researchers had reported the observation that the bone marrow and spleen contained cells of blood lineage that could rescue bone marrow pathology [7], it was studies by Till and McCullough in 1961 [8] and Moore and Metcalf in 1970 [9] that provided direct evidence that the bone marrow houses undifferentiated cells responsible for reconstituting the blood. As the primary mechanism that regenerates the cellular component of blood, hematopoiesis maintains key components of circulatory homeostasis.

Hematopoiesis relies on the maintenance of self-renewal and multilineage potential of a small population of quiescent stem cells in the bone marrow called

hematopoietic stem cells (HSCs) [10, 11]. When stimulated, HSCs proliferate to produce a daughter cell and a stem cell that becomes quiescent, thus maintaining the stem cell pool. Thus, while the majority of HSC progeny differentiate into hematopoietic progenitor cells (HPCs) that undergo successive periods of lineage commitment into the mature blood cells (Figure 1) [12], a few remain in the quiescent undifferentiated state, retaining their self-renewal and multipotency to maintain the HSC pool. HSCs therefore serve as a source of blood cells for the lifetime of the organism [13]. Maintenance of quiescence ensures that in the homeostatic state, HSCs maintain a very low proliferation rate to maintain blood and avoid premature bone marrow exhaustion [14, 15], while staying ready to respond to major hematopoietic stressors such as caused by severe injury or infection [5].

Through reconstitution experiments, it has been shown that the quiescent HSC pool in the bone marrow is a small population called long-term hematopoietic stem cells (LT-HSCs) or long-term reconstitution cells (LTRs) and that a single LT-HSC is capable of repopulating an entire hematopoietic system [16]. Mammals that have sustained disorders of hematopoiesis secondary to exposure to lethal and sublethal doses of radiation survive after bone marrow/HSC transplantation of donor cells [17, 18]. The transplanted donor HSCs migrate to the bone marrow, engraft to establish themselves in the marrow, self-renew and differentiate into blood cell lineages. HSCs maintain the ability of the hematopoietic system to keep the cellular component of blood in homeostasis throughout the lifespan of the organism. Due to their highly regenerative potential, HSC transplantation is the most widely used regenerative therapy clinically for the treatment of numerous blood disorders [6, 13, 19].

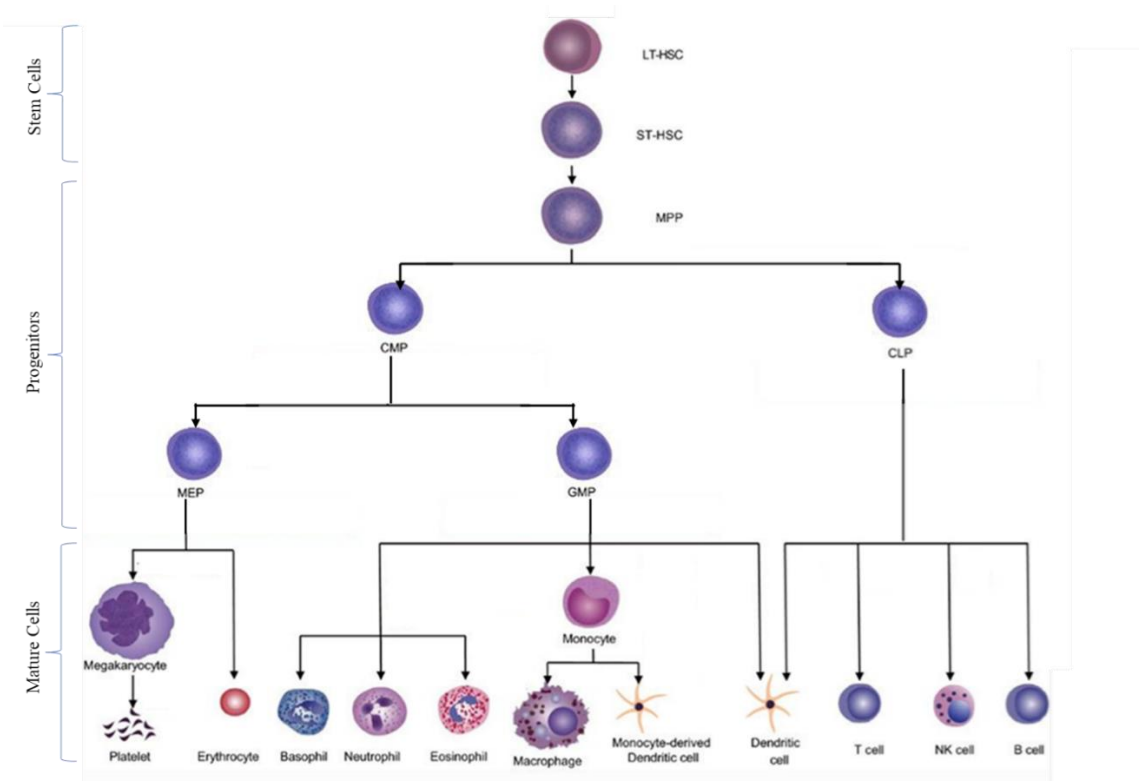

Figure 1: Hematopoietic tree. Schematic showing the classical model of differentiation and lineage commitment of hematopoietic stem cells (HSCs). While both long-term (LT-HSC) and short-term (ST-HSC) HSCs have multilineage potential, ST-HSCs have slightly reduced self-renewal capacity. ST-HSCs differentiate into multipotent progenitors (MPP), after which there is stricter lineage commitment into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMPs and CLPs can give rise to all mature blood lineages via several more lineage restricted progenitors such as, granulocyte-macrophage progenitor (GMP) and megakaryocyte-erythroid progenitor (MEP). Note: From "New paradigms on hematopoietic stem cell differentiation" by H. Cheng, Z. Zheng and T. Cheng, 2020, *Protein & Cell*, *11*, p. 36. Copyright 2019 by Springer Nature. Adapted with permission.

Ontogeny of Hematopoiesis in Mice

Development of hematopoiesis begins early in embryogenesis (Figure 2) [20]. Derived from the posterior and lateral plate mesoderm [10], the hematopoietic system is spatially and temporally tightly regulated in order to adequately serve the immediate physiological needs of the developing embryo while concurrently colonizing emerging

hematopoietic sites [5]. The first wave of hematopoiesis occurs extra-embryonically in the yolk sac at embryonic day (E) 7 [21]. Posterior mesodermal cells cluster in the yolk sac to form 'Blood Islands'; originally thought to be comprised of hemangioblasts-primitive cells that differentiate into early endothelial precursors (angioblasts) at the periphery of blood islands and primitive hematopoietic cells (erythroblasts) centrally [22, 23]. However, more recent evidence suggests that the emergence of primitive hematopoietic cells precede the formation of 'blood islands', where primitive erythroblast progenitor cells emerge from the posterior primitive streak and migrate towards the proximal yolk sac before being circumscribed by primitive angioblasts [24]. This early wave of hematopoiesis is only transient, ensuring a robust production of the much-needed erythroid cells early in development before the establishment of circulation. Primitive cells from the yolk sac at E7-E9 give rise to primarily primitive erythroid cells, primitive megakaryocytes, and myeloid cells (macrophages), and is termed 'primitive hematopoiesis' [21]. HSCs with higher hematopoietic potential appear in the yolk sac after E9, but have been shown to partly arrive from an intraembryonic source elsewhere, such as the aorta-gonad-mesonephros (AGM) [22].

Primitive hematopoiesis is followed by a second partially overlapping wave of hematopoiesis termed erythro-myeloid progenitor (EMP) wave. This wave is the first to generate definitive hematopoietic cells. At this stage in the yolk sac, definitive erythroid, myeloid, megakaryocyte, neutrophil, and mast cells emerge from erythro-myeloid progenitor (EMP) cells [25] derived from hemogenic endothelium. Near simultaneously, B and T lymphoid progenitor cells emerge in the yolk sac [26] and all the EMP and

lymphoid progenitor cells seed the fetal liver at E10 [27] prior to emergence of the third wave of hematopoiesis that procedes from the AGM region.

It is this third wave that gives rise to the first true HSCs. Definitive HSCs give rise to all mature blood cell lineages [28, 29]. Before the development of the fetal liver, only the AGM harbors definitive HSCs and at low frequency [30]. Murine AGM explants from E10 embryos clonally proliferate and differentiate into erythroid, myeloid and lymphoid lineages, as expected of definitive HSCs- a feature lacking in other hematopoietic embryonic tissues such as the yolk sac where EMP and lymphoid progenitors are largely independently produced [31, 32]. Thus, the AGM, specifically the dorsal aorta [33], is considered the first intra-embryogenic source of definitive HSCs. In what has been termed as endothelial-to-hematopoietic transition, specialized endothelium called hemogenic endothelium in the ventral wall of the dorsal aorta gives rise to these definitive HSCs [34-37]. At E10.5, definitive HSCs are also observed circulating in the umbilical and vitelline arteries between the AGM and placenta [38].

Another embryogenic organ that doubles as a hematopoietic organ is the placenta. Gekas et al. showed that HSC activity is observed in the placenta around E10.5-E11, paralleling the onset of hematopoiesis in the umbilical and vitelline arteries [38]. In vitro colony forming assays showed that the placenta harbors HSCs that generate hematopoietic progenitors with multipotent potential [39]. Ottersbach et al. showed that placental HSCs numbers increase rapidly between E11- E12 and possessed long-term reconstitution potential in irradiated hosts [40]. For this reason, it has been suggested that in addition to the AGM, the placenta partly contributes to definitive HSCs that colonize the fetal liver later [38].

From the AGM/placenta, definitive HSCs are seeded into the fetal liver (FL) around E11.5. HSCs from the FL are the most proliferative and are constantly cycling [41]. The self-renewal, differentiation and long-term repopulation potential of FL HSCs are higher than that of the bone marrow [42, 43] as the FL provides a microenvironment that promotes the rapid expansion of HSCs [4]. As the main hematopoietic organ in the developing embryo, the FL also provides an ideal microenvironment for the maturation of EMP progenitors and HSCs that arrive from the AGM and yolk sac [38]. Between E12 and E16, there is about 38-fold increase in expansion of HSCs seeded in the FL [44], indicating that the FL provides a niche uniquely suited for hematopoietic expansion unlike pre-FL hematopoietic tissues. It has been reported that the interaction between HSCs and $Nestin^{+}NG2^{+}$ cells in the FL drives this massive expansion [45], although cytokines are required to protect the cells from apoptosis [46]. After E16, the repopulation potential of HSCs from the FL declines as definitive hematopoiesis is being transferred to the developing bone marrow [47]. However, the FL remains the predominant hematopoietic organ in the developing embryo until about 3 weeks after birth in mice when environmental factors such as the expression of erythropoietin in the FL is suppressed [41].

By E12.5, HSCs are seeded into the fetal spleen, increasing gradually until around E17-E19 [48]. Although fetal spleen HSCs are definitive, they do not possess the high proliferative potential of FL HSCs. Although they are unable to maintain myelopoiesis initially [49], their long-term reconstitution potential increases with development [50]. Fetal spleen HSCs gain differentiation potential either while maturing in the spleen or through precursors that arrive from the FL [4]. Thus, the spleen primarily preserves the

self-renewal and differentiation potential of seeded HSCs [50]. However, the fate of these cells in the fetal splenic microenvironment is largely erythroid and myeloid [51]. The fetal spleen maintains active hematopoiesis until 14-15 days after birth [52].

Shortly before birth, around E16-E18 [52, 53], HSCs are seeded in the fetal bone marrow (FBM). Transfer of hematopoiesis to the bone marrow is the first step in ensuring a lifetime of continuous blood cell production. Although the bone marrow exists in all long and flat bones in the body, it is the FBM in the long bones that has been studied in detail. Similar to the spleen, the earliest HSCs that colonize the FBM at around E16 lack full hematopoietic functionality [54]. Full HSC activity is only detected in the FBM at E16.5, when the FBM is sufficiently vascularized and osteolineage cells have begun calcifying the marrow [55]. Interestingly, HSCs that arrive from other sources such as the fetal liver initially localize to the perivasculature in the developing diaphysis before the trabecular regions [55]. Functional long-term repopulation activity of FBM HSCs is significantly less that of the FL [56] and is only detectable around E17.5 [53], suggesting that the bone marrow microenvironment only then provides the necessary conditions for the maturation of the initial HSCs. Recently, Hall and colleagues [54] showed that while they do possess some hematopoietic activity, FBM HSCs do not show robust functional long-term repopulation until at the time of birth at P0. At about 3 weeks after birth, the newborn murine bone marrow becomes the dominant hematopoietic organ and unlike FL HSCs, the definitive HSCs in the bone marrow become quiescent from that point [41].

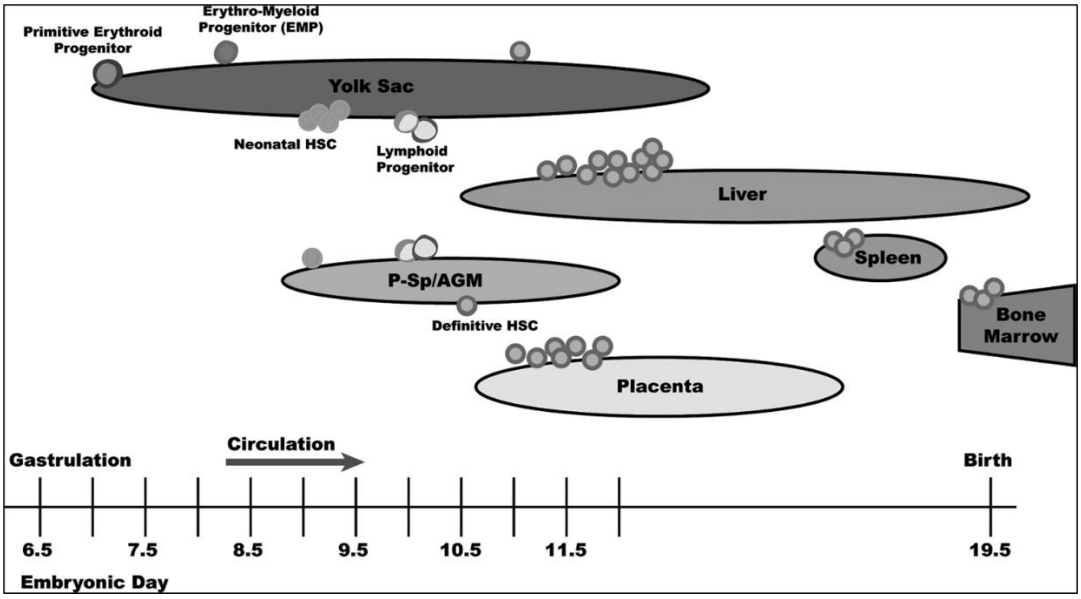

Figure 2: Ontogeny of hematopoiesis. Schematic showing the emergence of hematopoietic progenitors (circles) and the sequential changes in the sites of hematopoiesis (ellipses and trapezium) in the developing murine embryo over time. Primitive hematopoiesis begins around E7.25 in the yolk sac. Around E8.25, erythro-myeloid progenitors are detected in the yolk sac and later in the para-aortic splanchnopleure (P-Sp), followed by the emergence/detection of lymphoid progenitors. After E10, definitive HSCs are detected in aorta-gonad-mesonephros (AGM), yolk sac and AGM before being seeded in the placenta, fetal liver and then the fetal bone marrow . Note: From 'Lymphoid Progenitor Emergence in the Murine Embryo and Yolk Sac Precedes Stem Cell Detection' by Y. Lin, M. C. Yoder, and M. Yoshimoto, 2014, *Stem cells and development,* 23(11), p. 1172. Copyright 2014 by Mary Ann Liebert, Inc. Adapted with permission.

Hematopoietic niches

Hematopoietic cells exist in specialized microenvironments called niches, which represent unique anatomical sites that support the self-renewal, quiescence, differentiation and migration of stem cells [57]. In homeostasis, HSCs maintain blood through a balance between maintenance of quiescent HSCs and the differentiation of daughter cells into progenitors and precursors. Hematopoietic niches are 'designed' to support these highly regulated functions of HSCs [58]. In addition, niches with the right

conditions are required for the engraftment and homing of new stem cells and progenitors when they arrive through circulation. In disease states such as following irradiation, the blood system only survives when niches support (i) homing of transplanted HSCs into the bone marrow, (ii) HSC movement into 'healthy' locations within the marrow and (iii) their proliferation to repopulate HSC niches with adequate stem cells, differentiate into progenitors for each lineage in the marrow, and reconstitute blood [59].

The most well characterized hematopoietic niche is that of the adult bone marrow. For hematopoiesis to thrive in the adult bone marrow, the niche must provide the right conditions necessary to support the tightly regulated and metabolically demanding activities of stem cells in the marrow [60, 61]. Niches provide localized conditions, such as for oxidative phosphorylation, anaerobic glycolysis and calcium signaling essential for preserving HSCs and their function [62]. To meet these physiological demands, the niche houses supporting stromal/mesenchymal cells and the secreted soluble factors from these cells mediate self-renewal, differentiation, intra-marrow migration and mobilization of HSPCs, which also respond to hematopoietic demands via cell-to-cell interactions or cytokine signaling [63]. Hematopoietic niches exist in the bone marrow of all the long bones (such as the tibia and femur) and flat bones (such as the skull and vertebra) in the body [64, 65].

Within the bone marrow, HSC localization in homeostasis has recently been recognized as dynamic, potentially occupy multiple niches [66, 67]. However, only two main niches have been characterized- the endosteal niche and the vascular or perivascular niche (Figure 3) [68, 69]. The endosteal niche contains only about 10-20% of HSCs in the bone marrow. In this niche, HSCs are located within 10μm of the endosteum where

osteoblasts reside [68, 70]. Thus, in the endosteal niche, HSCs reside with predominantly osteoblasts and osteoclasts- bone cells which have been shown to play essential role in regulating HSC function [71]. For instance, in studies where osteoblast numbers were experimentally manipulated in the bone marrow, reduced HSC numbers correlated with reduced osteoblast numbers [72, 73].

In the vascular niche, however, HSCs localize in close proximity to the sinusoids and arterioles where their activity is influenced by the vascular and sinusoidal endothelium [41, 65]. The vascular niche contains the majority of HSCs in the bone marrow, with HSCs five times more likely to localize in the perivascular niche than the endosteal niche [67, 74]. About 80% of HSCs localize to the sinusoids and a further 10% to the bone marrow arterioles [70]. Also present at the vascular niche are supporting cells such as nestin+ mesenchymal stem cells [75], CXCL12-abundant reticular (CAR) cells [76, 77] and megakaryocytes [77, 78]. The dual components, the endosteal niche and the vascular niche, of the bone marrow makes the bone marrow unique in its organization compared to other hematopoietic niches in other hematopoietic organs. However, it is not entirely clear whether these two niches are true distinct HSC niches or they represent the transient intra-marrow migration of HSCs between the sinusoids and the endosteum [63, 65].

In addition to osteoblasts, endothelial cells, CAR cells and megakaryocytes, hematopoietic cells co-exist with other non-hematopoietic mesenchymal/stromal cells such as adipocytes[79] , chondrocytes [4] and sensory nerve endings [80, 81]. The role of non-hematopoietic cells on hematopoiesis has been shown to be largely through the secretion of important factors [82]. Both autocrine and paracrine processes are involved

in the production of hematopoietic regulatory cytokines and chemokines, which are frequently produced by non-hematopoietic cells including bone marrow stromal cells and endothelium [1, 83]. For example, endothelial cells and perivascular leptin receptor-positive ($Lepr^{+}$) stromal cells express stem cell factor (SCF), an important growth factor required for the maintenance and function of HSCs in the marrow [84]. SCF binds to its receptor C-Kit on HSCs to activate intracellular mechanisms that promote HSC maintenance [70]. Other niche cells such as osteoclasts secrete angiopoietin-1 that promotes HSC function [85]. The high amounts of calcium produced from osteoclastic activity promote HSC engraftment and maintenance [82, 86]. Osteoblasts secrete CXCL12 [87], thrombopoietin [88] and angiopoietin [89] that regulate HSC maintenance and function. Other perivascular cells, such as CXCL12-abundant reticular (CAR) cells, also influence HSC function via secretion of high amounts of CXCL12 [90]. CXCL12 binds to and activates its receptor CXCR4 on HSCs and other hematopoietic cells [91]. Activation of the CXCL12-CXCR4 pathway controls proliferation and retention of hematopoietic stem and progenitors in the marrow [70, 92]. However, signaling via direct cell-to-cell interactions between hematopoietic and non-hematopoietic cells through binding of receptors to their membrane-bound ligands has also been implicated [41].

A key regulatory element of hematopoiesis is neuronal control by intra-marrow sympathetic and sensory nerve endings [93-98]. Bone marrow neurons serve as chief integrators of signals from distant tissues and organs to regulate the bone marrow's response to distant stressors [99]. Majority of bone marrow nerves are perivascular, travelling parallel to the vessels, while a smaller population is observed at the edge of the

bone and in the marrow parenchyma around cells, forming a structural complex called the neuro-reticular complex with surrounding reticular cells [75, 93, 97].

HSPCs and other mesenchymal cells express adrenergic receptors that mediate their response to noradrenaline release within the marrow [99, 100]. For example, granulocyte colony-stimulating factor (G-CSF) mobilizes HSPCs through adrenergic stimulation and the circadian release of HSPCs is dependent on sympathetic stimulation [100, 101]. Catecholamines promote HSPC mobilization and egress out of the bone marrow [102]. Sensory stimulation is also critical to hematopoiesis. Sensory fibers in the bone marrow express the sensory neuropeptide calcitonin gene-related peptide (CGRP), and its receptor RAMP1 is expressed on HSPCs and some mature lymphocytes [103-105]. Recently, it has been shown that sensory stimulation is similarly required to enforce HSC egress out of the marrow and it may work hand-in-hand with the sympathetic nervous system [106]. In that study, it required dual denervation of sensory and sympathetic neurons to alter HSC dynamics in the bone marrow. Maintenance of HSC in the bone marrow is therefore a collaborative effort between sympathetic and sensory innervation.

Other important elements of the niche that affect hematopoiesis are the extracellular matrix (ECM) components including, collagens, fibronectin, heparin, and other ECM proteins. ECM provides a scaffold for the engraftment of HSCs and their progenitors in the marrow [1]. In the endosteal niches, extracellular calcium in the ECM released by bone cells activate calcium-sensing receptors (CaR) on HSCs, facilitating their homing to the niche [107]. In the vascular niches, CXCR4 activation on HSCs leads to expression of adhesion molecules such as E-selectin that facilitate their attachment to

collagen I in the ECM [107]. In addition to these structural components, the ECM contains trace elements, vitamins and other essential ligands that arrive in the marrow from the external environment [1].

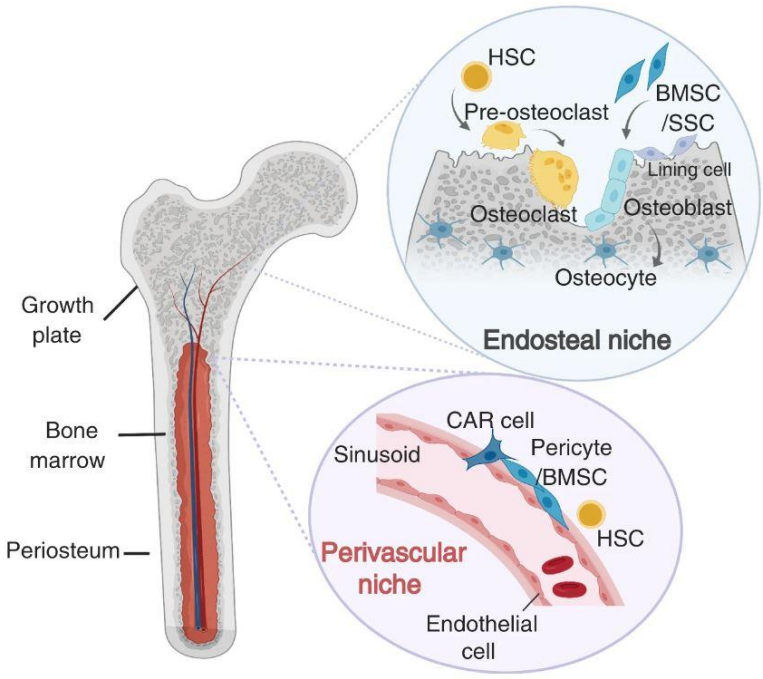

Figure 3: Hematopoietic niches. Schematic showing the endosteal (top circle) and perivascular/vascular niches (bottom circle) of the bone marrow (enlarged from the trabecular region). The endosteal niche comprises of HSCs co-inhabiting with the bone cells (osteoblasts and osteoclasts) that line the bone. The perivascular niche is located around the sinusoids populated by HSCs, endothelial cells and other perivascular cells such at CXCL12-abundant reticular cells (CAR) cells. Note: From 'Bone Marrow Niches in the Regulation of Bone Metastasis' by F. Chen, Y. Han and Y. Kang, 2021, *Br J Cancer,* 124, p. 1913. Copyright 2021 by Springer Nature. Reprinted with permission.

Bone marrow in calvarium

Development of the calvarium begins prenatally from skeletogenic mesenchymal cells derived from the neural crest and mesoderm [108]. Beginning around E9.5, the frontal calvarium is formed from the neural crest while the parietal originates from the

mesoderm. The two regions merge to form the rudimentary coronal and sagittal sutures and progress posteriorly to form the occipital calvarium [108]. By E12.5, osteogenic precursors migrate into the calvarium and facilitate the expansion of the calvarium apically and laterally [109]. By E14, osteogenic precursors have matured into osteoblasts which deposit collagenous extracellular matrix [110] and mineralize the calvarium to allow the successful engraftment of HSCs that arrive through circulation [86, 111, 112]. Despite the observation of HSCs in the calvarium before birth, unlike other fetal hematopoietic organs, development of hematopoiesis in the calvarium has not been well investigated.

In the 1990s, a group of investigators reported that newborn calvaria of mice lacking macrophage colony-stimulating factor (M-CSF) contained unique stromal cells that promoted differentiation into multiple hematopoietic lineages [113]. Named OP9 cells, coculture of embryonic stem cells with OP9 cells facilitated differentiation of the stem cells into erythroid, myeloid and B lymphoid lineages [114, 115]. This finding gave an indication of the potential uniqueness of the calvarial marrow but has gone relatively unexplored. However, much of our recent understanding of the organization of the hematopoietic niche and HSC engraftment have come from intravital imaging of the adult calvarial marrow [116, 117].

The adult calvarial marrow is entirely trabecular bone with cells and vasculature interspersed in between (Figure 4). In the calvarial marrow, it has been reported that while mature cells are distributed throughout the marrow, HSCs preferentially engraft around sinusoids (vascular niche) where there is increased expression of SDF-1/CXCL12 and E-selectin [118]. The bone marrow microvasculature is structurally and functionally

heterogenous, comprising of arteries, arterioles, veins, venules and sinusoids intricated together in a complex architecture [119, 120]. While arteries and venules deliver nutrients and remove wastes, sinusoids serve as conduits for mobilization of cells and as a niche for HSCs and megakaryocytes [119, 121]. The microvasculature of the calvarial marrow was first described in detail by Mazo and colleagues [122]. There is a distinct patterned network of sinusoids that drain into a central venule. The central vein runs parallel and close to the coronal suture. In addition, the lateral regions of the marrow cavities contain parasinusoidal and parasagittal vessels that also drain into the central collecting venules, as well as bone vessels that supply the bone itself. Herisson et al. [64] observed osteoblast-lined channels that connect the calvarial marrow to the brain dura beneath. These channels serve as conduits for blood vessels that connect the calvarial marrow to surrounding tissues like the brain. The channels also serve as exit route for hematopoietic cells out of the calvarial marrow into the brain [64, 123]. Pulous and colleagues observed that cerebrospinal fluid (CSF) exits into the calvarial marrow through the perivascular spaces of these channels [124]. Thus, the transcortical channels may serve as a route of communication between the calvarial marrow and the central nervous system via the CSF.

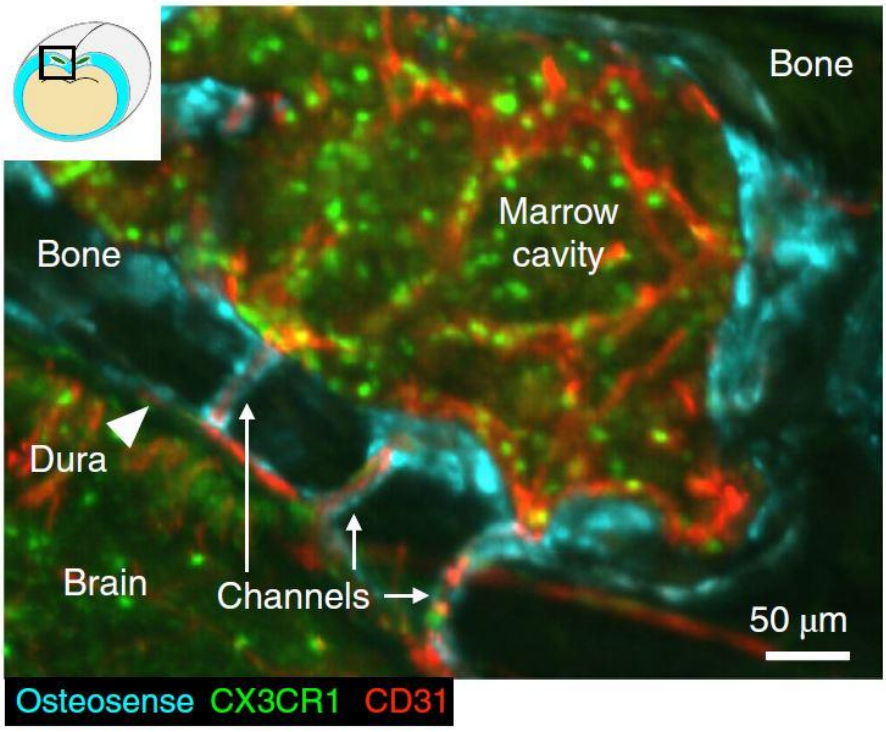

Figure 4: The bone marrow in the calvarium. Representative image of coronal section through intact skull and brain of a mouse showing the bone marrow in the calvarium. The trabecular marrow cavity bounded by bone (Osteosense, cyan) is filled with hematopoietic cells (CX3CR1+ cells, green) with interspersed vasculature (CD31+, red). The calvarial marrow cavity (top) is connected to the brain dura (bottom) by vascular channels (arrows). Note: From 'Direct Vascular Channels Connect Skull Bone Marrow and the Brain Surface Enabling Myeloid Cell Migration' by F. Herisson et al., 2018, *Nat Neurosci.* 21, p. 1214. Copyright 2018 by Springer Nature. Reprinted with permission.

Bone marrow in long bones

Development of the long bones begin prenatally, just about when definitive hematopoiesis has begun [125, 126]. Around E9 and E10, for the fore limbs and hind limbs respectively, the limb buds appear in the lateral wall of the mesoderm, from which mesenchymal cells proliferate and differentiate into the rudimentary limbs [127]. While HSCs are seeded in the developing long bones around E16 [53], they do not mature until after birth [54]. The adult bone marrow in the long bones is the bone marrow

compartment that has been extensively studied. Unlike the flat bones of the calvarium, the long bones are comprised of trabecular bone (at the proximal and distal epiphyses and metaphyses) and a hollow compact bone (diaphysis) (Figure 5) [128, 129]. Interspersed within both trabecular and compact regions are cells and vasculature. Histological investigations have revealed that unlike mature hematopoietic cells which are dispersed throughout the marrow, majority of HSCs in the long bones reside in the trabecular regions [41, 130, 131] where they may be influenced by factors secreted by osteoblasts and osteoclasts [67]. However, similar to the calvarial marrow, HSCs preferentially localize in close proximity to the vasculature and sinusoids (vascular/ perivascular niche) in long bones, whether in the trabecular regions or in the diaphysis [67, 120, 132]. Interestingly, Guezguez and colleagues [132] found that HSCs that localize to the trabecular regions showed higher self-renewal and regenerative potential compared to those that localized to the diaphysis.

The long-bone marrow microvasculature also shows a unique vascular organization [80]. As described by Nombela-Arrieta et al. [120] using a 3D imaging setup, there is a central large artery which courses through the center of the diaphysis, branching into smaller arterioles towards the bone edges. These arterioles partly transition into venules at the endosteum while also giving rise to the sinusoids that course back towards the center of the diaphysis, and venules drain into a central sinus [80]. Li et al. [121] observed a similar organization in the diaphyseal region, in which the arteries show a tree-like pattern towards the center of the diaphysis while the sinusoids, in a complex network, make up the majority of vasculature in the marrow. The microvascular organization of the trabecular region has less structure, with a number of arteries entering

the marrow through the bone cortex and branching into smaller arterioles along the endosteum. The arterioles course towards the center of the marrow where they transition to the sinusoids in the process. There are fewer transitional vessels in the trabecular zone compared to the diaphysis [121].

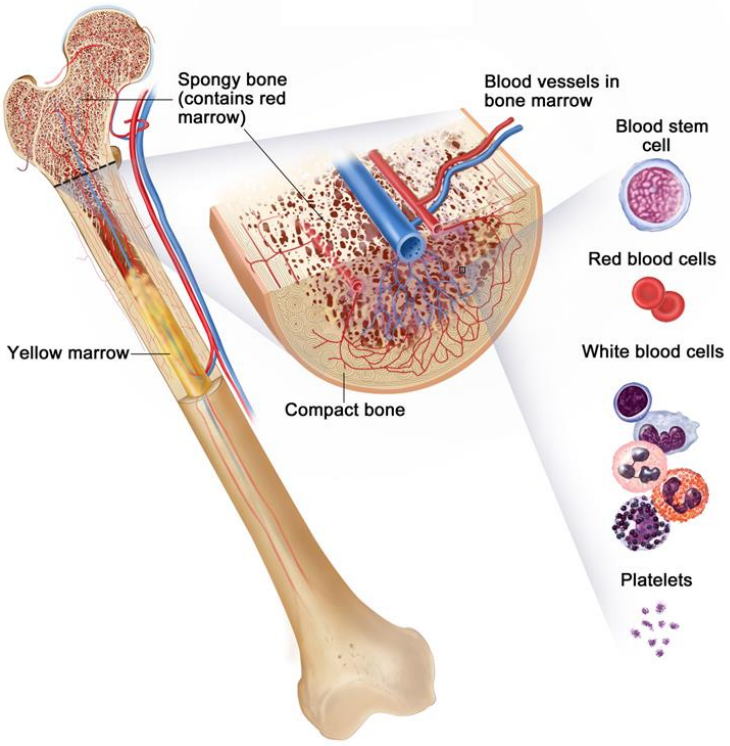

Figure 5: The bone marrow in long bones. Schematic showing the bone marrow in femur. The distal parts of the bone are entirely trabecular (spongy) while the middle diaphysis is hollow, filled with marrow. 'Red marrow' becomes 'yellow marrow' with age due to deposition of fats. Inside the marrows, all blood cell lineages are generated from stem cells. Note: From "Bone Anatomy" by Terese Winslow, 2014*, National Cancer Institute Visuals Online*. Copyright 2014 by Terese Winslow. Reprinted with permission.

Bone marrow-derived cells in retinal injury

The bone marrow serves as a readily available source of inflammatory and reparative cells to the retina and the rest of the body. In addition to HSCs, the bone

marrow also houses mature cells and progenitors for circulating/myeloid angiogenic cells, neutrophils, monocytes, macrophages, microglia and lymphocytes [133-138]. Studies have shown that bone marrow-derived progenitors can travel to peripheral ischemic sites to support restoration of tissue health or in disease can contribute to long-term pathophysiology [139-142]. In the retina, acute and chronic injury lead to mobilization and recruitment of bone marrow-derived hematopoietic cells and their transmigration into the parenchyma [143-145].

When the retina or other peripheral tissues are injured, cytokines and other regulatory factors are released into circulation, which triggers the mobilization of bone marrow-derived cells from the marrow and their subsequent homing to the sites of injury [146, 147]. Inflammatory signaling cascades that lead to activation of receptors on bone marrow cells are triggered by damage-associated molecular patterns (DAMPS) produced from the injury [148]. DAMPS facilitate recruitment of bone marrow inflammatory cells by activating toll-like receptors (TLRs) on these cells [149]. Also, hematopoietic progenitor cells can be mobilized into peripheral tissues via activation of adhesion molecules and metalloproteinases (MMPs) [139]. Growth factors such as placental growth factor (PGF) and vascular endothelial growth factor-A (VEGF-A) induce MMP expression in the bone marrow, which activates the migration of progenitors towards the sinusoids and out into circulation [150, 151].

Once in the retina, bone marrow-derived cells collaborate with resident cells to facilitate tissue repair or promote pathophysiological events that propagate a worsening retinal disease [143]. Leukocytes including neutrophils, monocytes and MACs participate in the milieu that proceeds tissue injury. In response to inflammation, the endothelium

secretes adhesion molecules such as vascular cell adhesion molecules (VCAMs) and intercellular adhesion molecules (ICAMs) that make the vascular walls at the injured site "sticky" with adhesive ligands to attract circulating cells [152]. Leukocytes, which also express cell adhesion molecules, adhere to vascular endothelium and cause leukostasis [153, 154]. The ensuing milieu of capillary obstruction, microaneurysm [155] and production of more inflammatory agents leads to blood-tissue barrier breakdown and the eventual transmigration of the leukocytes into the retina[152, 156-159]. Once in the retina, the major mechanism by which these cells participate in the retina's response to injury is through paracrine secretions [143]. For tissue repair, bone marrow-derived cells secrete factors that suppress inflammation and apoptosis and promote angiogenesis and cell survival [160]. In the damaged retina, monocytes secrete cytokines that promote the inflammatory response and later differentiate into macrophages that survey the retina and facilitate healing by phagocytosing dead cells [161, 162]. Neutrophils, which are the first to respond to tissue injury, increase neuronal survival by expressing crucial paracrine proteins [163]. In addition, bone marrow-derived MACs closely surround damaged vessels and neovasculature [164, 165]. MACs secrete paracrine factors that promote vascular repair and angiogenesis [166].

In addition to tissue repair, bone marrow-derived cells harbor the potential to worsen tissue injury and disease through the release of pro-inflammatory cytokines [167, 168]. For example, it has been shown that following ischemic injury, tissue and serum levels of inflammatory cytokines such as interleukin-6 (IL-6) increase, attracting inflammatory cells to the site of injury. IL-6 then activates IL-6 receptor on bone marrow-derived monocytes/macrophages, which in turn produce more IL-6 to recruit

more inflammatory cells [147]. In the bone marrow, IL-6 together with IL-3 stimulate the acute proliferation of hematopoietic progenitors and their release into circulation [169, 170]. Thus, as the initial tissue repair response fails, inflammatory cytokines activate microglia and macrophages, which also secrete more neurotoxic inflammatory cytokines that worsen the pathology.

The bone marrow in diabetes

Diabetes is a complex metabolic disorder characterized by chronic hyperglycemia, hyperinsulinemia and dyslipidemia [171-177], leading to severe multi-organ complications in the eye, kidney, heart, among others [178]. Like vital organs, current literature recognizes the bone marrow as another target of diabetic end-organ damage [99], where diabetes-associated metabolic dysfunction leads to a disruption of the balance between inflammatory damage and reparative mechanisms. Diabetes-associated bone marrow damage is largely responsible for the impairment of the body's cellular-based reparative mechanisms [99, 178-180].

Diabetes end-organ damage in the bone marrow occurs in multiple ways. First, there is microangiopathy- loss of the integrity of the local bone marrow microvasculature [99]. Outside the gut, vascular endothelial cells are the first cell types in any organ that immediately encounter and respond to abnormally high levels of circulating blood glucose [181, 182]. The bone marrow contains arterioles, venules and capillaries with tight vascular barrier that controls trafficking of molecules in and out of the marrow. The sustained exposure of endothelial cells in these vessels to high glucose leads to impaired cellular function [182] and subsequent inflammatory response [183, 184]. There is a breakdown of the vascular barrier established by endothelial cells in the marrow [185]

and the uncontrolled entry of inflammatory molecules leads to dysfunction of the sinusoids, small vessel rarefaction and a disruption of the vascular niche [99]. There is reduction in proportion of total hematopoietic cells, HSPCs and mislocalization of HSCs [179]. In addition, sinusoidal endothelial cells are depleted while endothelial progenitors are dysfunctional, with reduced ability to proliferate and form networks [179]. These abnormalities have been attributed to reduced perfusion and supply of nutrients as well as increased oxidative stress in the marrow [185].

In correlation with microangiopathy, diabetes leads to bone marrow neuropathy, in which there is a general loss of sympathetic nerve fibers over time in the long bone marrow [99]. There is strong evidence in the literature that impaired HSPC mobilization and function is largely due to loss of sympathetic innervation in the bone marrow [186]. Busik et al. [187] and Albeiro et al. [186] observed a reduction in sympathetic nerve endings in the bone marrow in diabetic rodents and humans. However, another study found increased sympathetic innervation in the calvarium in rodents [188], suggesting that different bone marrow sites may exhibit different responses to diabetic metabolic disorder.

Closely related to bone marrow neuropathy is impaired HSPC function [186]. HSPCs from diabetics show abnormally increased oxidative stress and apoptosis- features of abnormal molecular integrity as a result of poor perfusion [185]. The lack of nutrients and important trophic factors destabilizes the bone marrow microenvironment [189], the vascular niche in particular, with negative consequences on hematopoietic homeostasis [185, 190]. In homeostasis, HSPCs are mobilized into circulation where they can exert their reparative functions on distant tissues. Efficient mobilization requires HSPC

proliferation and the activation of MMPs to enhance cell movement and egress out of the marrow [100]. Diabetes suppresses the proliferation and adhesion ability of HSPCs, impairing their ability to support vascular repair [191]. In addition, there is impaired mobilization of HSPCs out of the marrow into circulation [192], which is believed to be responsible for impaired vascular repair in peripheral tissues such as the retina, kidney, heart and other organs of the cardiovascular system [187, 188, 193, 194].

Diabetic HSPC mobilopathy has been attributed in part to poor response of HSPCs to mobilizing agents such as G-CSF and a subsequent inability of niche supporting cells to downregulate the stem cell retention factor CXCL12 [180, 188]. G-CSF stimulates HSPC mobilization via sympathetic activation, and sympathetic norepinephrine release activates adrenergic receptors on niche cells to rapidly downregulate CXCL12 expression [195, 196]. Thus, bone marrow mobilopathy is largely associated with local neuropathy.

In addition to HSPC mobilopathy, chronic diabetes drives myelopoiesis- the proliferation and migration of leukocytes into circulation [197-201]. Myelopoiesis has been shown to be closely linked to HSPC mobilopathy [202, 203]. Within the bone marrow, hematopoiesis follows a hierarchy, whereby HSCs divide and differentiate through stages into myeloid, erythroid and lymphoid lineages [198]. Diabetes skews the differentiation of the hematopoietic progenitors towards the myeloid lineage, leading to leukocytosis [204, 205]. There is increased expression of damage-associated molecular patterns (DAMPS) such as S100A8/s100A9, which activate receptors for advanced glycation end products (RAGEs) on hematopoietic progenitors [206] thereby driving their differentiation into inflammatory myeloid cells such as neutrophils and monocytes [204,

207]. Also, DAMPS induce increased expression of cytokines such as IL-1β by activating TLR4/MyD88 pathway. IL-1β activates its receptors on HSPCs to drive their differentiation into monocytes and neutrophils [208]. Thus, diabetes alters the hematopoietic niche [209] by inducing the expression of DAMPS and inflammatory cytokines, which activate myeloid differentiation pathways in hematopoietic progenitors leading to myelopoiesis.

Diabetes is also associated with uncontrolled accumulation of fats/lipids in the bone marrow [210]. The number and size of adipocytes in the bone marrow increases in type 2 diabetic mice, and this negatively correlates with HSC activity as excess adipogenesis inhibits homing of HSCs and slows down hematopoiesis [211]. Long-standing diabetes alters the differentiation potential of bone marrow mesenchymal/stromal stem cells [212], skewing their differentiation away from osteoblast and chondrocyte lineages and towards adipocyte lineages to enhance adipogenesis [213-215]. Botolin and colleagues observed an increase in adipocyte markers as well as increase in the number of adipocytes in the tibias of diabetic mice compared to controls, while osteoblastic markers were decreased [216]. Elevated bone marrow adipogenesis in diabetes has been shown to be mediated by high glucose-associated upregulation of adipocyte transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and the CCAAT/enhancer-binding protein alpha (C/EBPα) [217, 218]. Elevated serum lipids during dyslipidemia also stimulate increased expression of PPARγ and inhibit osteoblast differentiation [219], facilitating enhanced adipogenesis in the bone marrow.

Concluding Remarks

As described in the preceding subsection, the bone marrow is an important target of metabolic disorders such as diabetes [99]. It has been hypothesized that mobilopathy of vascular reparative cells from the bone marrow is a significant contributor to the microangiopathy observed in diabetic retinopathy [180]. However, bone marrow exists in different types of bones in multiple locations within the body and it is not known whether the response of the marrow in these different compartments to retinal injury is homogenous. Understanding how hematopoiesis in different bone marrow compartments differs in development and in response to retinal injury is critical to our understanding of hematopoietic regulation with implications for future advances in cell-based therapies for diabetic retinopathy. The subsequent sections in this dissertation will explore hematopoietic development in different marrow locations and its relevance to retina development. They will also cover how acute retinal injury engages the hematopoietic system in the long bones such as the tibia compared with that of flat bones such as the calvarium and how these different bone marrows respond to chronic metabolic insults. This work will help identify novel, mechanism-based approaches for retina repair that can cure diabetic retinopathy, rather than only addressing the symptoms. It is well known that diabetic retinopathy occurs later during diabetes, typically after 20 years in humans despite the presence of hyperglycemia throughout this time. However, why this delay occurs is poorly understood. This work provides a paradigm shift by proposing that the delay is due to the ability of the calvarial marrow to provide functional myeloid angiogenic cells (MACs) for vascular repair. The studies provide support that until late in the disease course the calvaria is resistant to hyperglycemic damage because of the

unique communication it has with cerebrospinal fluid which can provide it with neurotrophic and growth factor support and sustain hematopoiesis.

DEVELOPMENTAL DELAY IN CALVARIAL HEMATOPOIESIS AND THE DIFFERENTIAL RECRUITMENT OF BONE MARROW-DERIVED CELLS INTO THE INJURED RETINA

by

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ABSTRACT

The hematopoietic system produces the cellular components of blood and it is the predominant system that generates the inflammatory/immune cells that invade injured and diseased tissues. Seeded by circulating hematopoietic stem and progenitor cells shortly before birth, the bone marrow becomes the primary hematopoietic organ in the adult state and resides in all long and flat bones in the body. Studies have shown that injury and disease alters the homeostatic state of hematopoiesis, leading to the generation and mobilization of both inflammatory and reparative cells from the bone marrow to target tissues. Whereas extensive studies have been performed on the development of hematopoiesis in the long bones, the process of hematopoiesis in the calvarium is incompletely understood. In this study, we compared the development of hematopoiesis in the calvarium and long bones postnatally in mice and how the different bone marrow sites influence mobilization of cells following acute retinal ischemic injury. We show that hematopoiesis is developmentally delayed in the calvaria marrow compared to the long bones until P21, when both compartments show robust hematopoiesis and the bone marrow becomes the predominant hematopoietic organ in the body as the liver's role subsides. Using the competitive repopulation assay, we show that cells of long bone origin reconstitute blood faster than those of calvarial origin. However, the calvaria marrow provides a microenvironment that promotes engraftment of hematopoietic stem and progenitor cells. In addition, we show that the calvaria marrow contributes more neutrophils and MACs to the retina than the long bones during acute ischemic retinal injury, even though the proportion of neutrophils and MACs at baseline are not significantly different between the two compartments.

Keywords: calvarial marrow, long bones, calvarium, HSPCs, ischemia-reperfusion, neutrophils, myeloid angiogenic cells

INTRODUCTION

The hematopoietic system, the innate machinery that ensures consistent production of erythroid, myeloid and lymphoid cells, repopulates the cellular component of blood [1-4] and supplies the inflammatory cells that home to injured tissues [5-7]. Hematopoiesis arises from HSCs- a small population of normally quiescent multipotent cells that maintain self-renewal and multilineage potential. In mice, hematopoietic cells first appear in the yolk sac at embryonic day (E)7 in what is termed as 'primitive hematopoiesis', prior to the emergence of the first definitive HSCs in the AGM at E10 [8, 9]. The cells then migrate to the fetal liver, spleen and bone marrow at E11.5, E12 and E16-E18 respectively [10]. The fetal liver dominates blood cell production prenatally and in the newborns until shortly after birth when the bone marrow becomes the dominant hematopoietic organ [3, 8, 11]. Expansion and subsequent differentiation of HSCs upon stimulation generates lineage-committed progenitors [12] that give rise to mature blood cells such as erythrocytes, monocytes, neutrophils, and lymphocytes that are released into circulation [13, 14]. Even in the healthy state, the survival of the hematopoietic system depends on the ability of bone marrow long-term hematopoietic stem cells (LT-HSCs) to maintain their ability to produce circulating myeloid and lymphoid cells to replace those cells that complete their lifespan or die by apoptosis [15].

It is well documented in the literature that both acute [7, 16-18] and chronic [16, 19, 20] retinal injury result in recruitment and homing of bone marrow-derived cells into the retina. Injury to peripheral tissues like the retina leads to the release of cytokines and regulatory factors into circulation that initiate the mobilization of bone marrow-derived cells out of the marrow and their subsequent homing into the sites of injury [21, 22].

DAMPS released from injured tissues trigger inflammatory signaling cascades that activate receptors on bone marrow cells [23]. Myeloid cells such as neutrophils, monocytes and MACs have all been implicated in the milieu that follows acute and chronic retinal injury. In response to inflammation, neutrophils, the earliest responders, adhere to the retinal endothelium and cause leukostasis, resulting in capillary obstruction, microaneurysms, blood-retinal barrier breakdown and exudation [24-27].

These pathophysiological processes facilitate the transmigration of activated inflammatory cells into the retina [28]. Infiltrating monocytes enter the retina from multiple sources including the optic nerve head [29], RPE/choroid [30, 31] and the retinal vasculature as patrolling monocytes [29]. They initially secrete cytokines that further the inflammatory response, then transmigrate and differentiate into macrophages in later stages [29, 32] to facilitate healing by phagocytosing dead cells [17]. MACs, previously known as circulating angiogenic cells (CACs), are bone marrow-derived cells that help preserve vascular homeostasis [33, 34]. When recruited into the injured retina, they facilitate vascular repair by paracrine secretion of cytokines supportive of retinal endothelial cell function [35, 36].

Hematopoietic cells exist in microenvironments housed and protected by bones [8]. The microenvironments have a complex organization, comprising of a vascular niche (near the sinusoids, where most HSCs localize) and an endosteal niche (near the bone endosteal lining) that support and maintain HSC function [11, 37]. These niches have been shown to exist throughout the body in flat bones such as the calvarium [38-43] and long bones such as the tibia [44-48]. The calvarial and tibial marrows release cells into periosteal circulation via a network of vascular channels that traverse the bone [38, 49].

Despite the general appreciation of the existence of hematopoietic cells in these distinct locations, the physiological differences between these sites have not been extensively studied. The development of hematopoiesis in the calvarium, which matures later than the long bones, and how it differs from that of the long bones, is incompletely understood. In addition, the differences in contribution of these sites to bone-marrow derived cells in the injured retina have not been investigated.

In this study, we compared the development of hematopoiesis in the calvaria marrow to the long bones postnatally. We show that the ability of seeded hematopoietic stem cells to expand and populate the developing marrow is delayed in the calvarium compared to the long bones. Also, we observed that reconstitution of the blood by calvaria marrow-derived cells is delayed compared to that of cells from long bones. However, the calvarium offers a microenvironment supportive of hematopoietic stem and progenitor cell engraftment. In addition, we identified that more neutrophils and MACs are mobilized from the calvarium into the retina following acute retinal ischemia.

METHODS

Animals

Four-to-eight-week-old CD45.1 (B6.SJL-Ptprca Pepcb/BoyJ) and C57BL6/J male and female mice were purchased from Jackson Laboratory (Jackson Laboratories, Bar Harbor, ME). CD45.1 mice were bred in-house to generate P7, P14 and P21 donor newborn mice while the adult C57BL6/J (CD45.2) mice were used as hosts for competitive repopulation assay. Four-to-eight-week-old KIKGR (Tg(CAG-KikGR)33Hadj/J) mice were purchased from Jackson Laboratory (Jackson Laboratories, Bar Harbor, ME) for in vivo bone marrow-derived cell labelling experiments. The animals were housed in a standard laboratory environment and maintained on a 12-hour light–dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham (APNs 21261 and 21291) and adhered to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Visual Research.

Isolation of bone marrow cells from tibia and calvarium

To ensure all tissues were treated in a similar manner, bone marrow cells were obtained by bone crushing. Briefly, animals were euthanized and the tibia and calvaria were carefully dissected from the legs and skulls respectively. Bones were cut into pieces with surgical scissors and then gently crushed for up to 1 minute with a mortar and pestle 3 times in 1ml FACS buffer. Crushed bones were filtered into a 50ml conical tube through a 40µm strainer, centrifuged at 350g for 5 minutes and the supernatants were discarded. Cell pellets were resuspended and red blood cells were lysed in 1-2ml ACK

lysis buffer. The cells were washed twice with FACS buffer and resuspended for subsequent experiments.

Flow Cytometry

For bone marrow-derived cells, single cells in suspension obtained as described above were incubated with a cocktail of primary antibodies for 45 min at room temperature in the dark.

For the retina, single cell suspensions were prepared for flow cytometry as previously published [31]. Briefly, mice were perfused transcardially under isoflurane anesthesia with phosphate-buffered saline (PBS). After euthanasia, eyes were enucleated into PBS on ice. The retinas were immediately isolated after removing the corneas and lenses. Each retina was incubated in 1 ml of papain dissociation solution (Roche Diagnostics GmbH, REF#10108014001, Mannheim, Germany) prepared according to the manufacturer's instructions for 30 min in a 37 °C water bath for dissociation into single cells. After washing with FACs buffer, single cell suspensions were incubated with primary antibody cocktails for 45 min in the dark.

Antibodies used included anti-Mouse Hematopoietic Lineage-e450 (Invitrogen, Cat# 88-7772-72), C-Kit-APC (BD Biosciences, Cat# 553356), Sca-1-BV605 (Invitrogen, Cat# 64-5981-82), CD45.1-BV650 (BioLegend, Cat # 110743), CD45.2-Percp-Cy5.5 (BD Biosciences, Cat# 552950), CD34-PE (Invitrogen, Cat# MA5-17831), Ly6C-Percp/Cy5.5 (Biolegend, Cat# 128012), Ly6G-BV605 (Biolegend, Cat# 127639), , Flt3-APC (Invitrogen, Cat# 17-1351-82), CD11b-BV786 (Invitrogen, Cat# 78-0112-82), CD45-APC-e780 (Invitrogen, Cat# 47-0451-82), CD31-PE (Invitrogen, Cat# 2114546),

Fixable Viability Dye eFluor506 (Invitrogen, Cat# 65-0866-14), CD45-BV650 (Invitrogen, Cat# 47-0451-82), CD31-BUV563 (BD Biosciences, Cat# 741251) and Flk-1 AF700 (Invitrogen, Cat# 56-5821-81). The cells were then washed with FACs buffer, resuspended, and analyzed using BD FACSCelesta flow cytometer (BD Biosciences) and FlowJo™ v10.8 Software (BD Life Sciences, Franklin Lakes, NJ, USA).

Competitive Repopulation Assay

The competitive repopulation assay was modified according to previously published protocols [50, 51]. Briefly, CD45.2 host mice were lethally irradiated with two doses of 4.50Gy total body gamma radiations four hours apart. Donor CD45.1 pups were euthanized and their calvaria and legs were harvested, cleaned, and rinsed in sterile PBS. While maintaining a sterile environment, tissue samples were digested with 5mg/ml collagenase I (Millipore Sigma, Cat# SCR103) at 37°C for 30 minutes. Samples were filtered through a 40μm strainer to obtain bone marrow cells in suspension. Samples were enriched for hematopoietic stem and progenitor cells using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (STEMCELL Technologies, Cat# 19856) according to manufacturer's instructions. A total of 1×10^6 cells in a 100μL volume was transplanted into each irradiated host retro-orbitally. Host mice were given antibiotics (Baytril, Bayer Healthcare LLC, Kansas, USA) orally in drinking water for two weeks post-transplantation as prophylaxis against bacterial infections. Host mice were bled retro-orbitally biweekly, starting 3 weeks post-transplantation, to enumerate the donor cells reconstituting the blood by flow cytometry. Host mice were euthanized 16 weeks post-transplantation and their calvaria and tibial bone marrows were harvested to assess the stem and progenitor cells in the bone marrows by flow cytometry.

Bone marrow photoconversion

KIKGR transgenic mice were anesthetized with a combination of ketamine (80mg/kg) and xylazine (15mg/kg). The fur covering the skulls and tibias was shaved and the skin disinfected with 70% ethanol. For the calvarium, a vertical incision was made in the skin above the sagittal suture and extended to the end of the occipital calvarium posteriorly and the frontal calvarium anteriorly. Extra membranes covering the skull were gently excised to allow the flexible movement of the skin. The two halves of the skin above the calvarium were then held apart to expose the entire calvarium and kept in place with tape. After ocular lubricant was placed on the eye, the front part of the head including both eyes were covered with aluminum foil held in place with tape. Extra precaution was taken to avoid photoconversion of surrounding tissues by covering the remainder of the body with aluminum foil.

For the tibia, a small incision was made in the skin on the medial side of the tibia and extended to the beginning of the patella and the distal tibia. Under a dissecting microscope, the muscle tissue beneath the skin was excised to expose the tibia while the leg was held in place with a tape. To avoid photoconversion of surrounding tissues, the rest of the body was covered with aluminum foil.

To photoconvert the bone marrow cells, the anterior (3 min) and posterior (3 min) halves of the exposed bones were illuminated by a 405nm laser fitted with a 5mm-diameter focusing lens (Dymax Bluewave QX4 V2.0, Dymax Corporation, Torrington, CT) at 75% power (16.5W/cm^2) for a total laser duration of 6 minutes. The focusing lens was positioned at 5cm from the surface. After photoconversion, the skin incision was closed using vetbond tissue adhesive.

Acute retinal ischemia/reperfusion injury (I/R)

Retinal I/R procedure was modified as previously published [52, 53]. Under deep isoflurane anesthesia, right eyes were cannulated with a 30-gauge needle connected to a sterile saline infusion bag to elevate the IOP (80-90mmHg) for 1 hr. Whitening of the iris and loss of red reflex confirmed retinal ischemia. After 1 hr of ischemia, the needle was removed, and the eyes were allowed to reperfuse. Mice were euthanized after 6 hrs.

CXCL12 ELISA

Bones were cut into pieces with surgical scissors and then gently crushed for up to 1 min with a mortar and pestle 3 times in 1ml FACS buffer containing protease inhibitors. Crushed bones were filtered through a 40µm strainer on top of a 50 ml conical tube. Samples were then centrifuged at 350g for 5 minutes and the supernatant collected by pipetting on ice. Samples were analyzed for CXCL12 levels using the Mouse CXCL12/SDF-1 DuoSet ELISA kit (R&D Systems, Cat# DY460) according to manufacturer's instructions.

Statistical Methods

Statistical analyses were performed using GraphPad Prism v9.1 software. All data data were assessed for adherence to normal distribution by the Shapiro-Wilks normality test. Data which conformed to normal distribution were analyzed by unpaired Student's t-test and data which failed to meet the assumptions of normality were analyzed using unpaired Mann-Whitney test. P-values less than 0.05 were considered statistically significant.

RESULTS

Hematopoietic cells in the calvarium and long bones during postnatal development

Although the fetal liver remains the dominant hematopoietic organ in the developing mouse embryo until shortly after birth [54], development of both the calvarium [55, 56] and long bones which house the bone marrow starts prenatally [57-59]. However, our understanding of hematopoiesis in development has largely been restricted to that occurring in long bones. We were interested in comparing the development of hematopoiesis at both marrow sites by determining if the proportions and function of hematopoietic cells were comparable at three key time points. We selected P7, when the fetal liver is still the dominant hematopoietic organ, P14, when hematopoiesis in the fetal liver is shut off allowing the bone marrow to begin to dominate and P21 when the bone marrow is the dominant hematopoietic site in the body [60]. We assessed the proportion of hematopoietic stem and progenitor cells (HSPCs) as well as mature (CD45+) cells by flow cytometry.

As shown in Figures 1 and 2, the proportion of HSPCs in the calvarium was significantly higher than that of the long bones at P7 and P14 but not at P21. Conversely, the proportion of mature cells in the calvarium was significantly lower than that of the long bones at P7 and P14 but not at P21. This suggested that the proportion of HSPCs were higher in the calvarium at P7 and P14 because of relatively low content of mature cells in the calvarium. This difference was lost at P21 when the cells in the calvarium had proliferated and differentiated to levels similar to those in the long bones.

Functional Hematopoiesis in the calvarium and long bones during postnatal development

Next, we tested the reconstitution ability of hematopoietic cells from these two locations using the competitive repopulation assay. The competitive repopulation assay is a gold standard technique for evaluating the self-renewal and multilineage differentiation potential of cells of hematopoietic origin in vivo [2], allowing assessment of short-term and long-term reconstitution of the host's bone marrow and blood by donor bone marrow cells. At P7, P14 and P21, isolated cells from either the calvarium or long bones were transplanted into lethally irradiated host mice and the hosts were bled biweekly to assess the repopulation of the blood by donor cells using flow cytometry.

As shown in Figure 3, when donor cells were obtained from P7 pups from either compartment [calvaria (red line) or long bones (green line)] the number of CD45.1 cells in the peripheral blood was the same by 6 weeks. However, the calvaria-derived donor cells were slower to repopulate the CD45.2 mice compared to the long bones. This same pattern was observed when transplanted cells were obtained from P14 pups or P21 pups. Thus, this data indicates that the HSPCs from long bones can repopulate a lethally irradiated host faster than HSPCs from the calvaria.

Next, we were interested in assessing the proportions of long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and multipotent progenitors (MPP) in the calvaria and tibias of stably transplanted host mice 16 weeks post transplantation. As shown in Figure 4, for both groups of mice (calvaria hosts and tibia hosts) that received P7 donor cells, there was no significant difference in the proportions of LT-HSCs, ST-HSCs and MPPs. However, we observed significantly higher proportions of LT-HSCs, ST-HSCs and MPPs in the calvaria than tibias in mice that received P14 and P21 calvarial donor

cells. Similarly, for mice that received tibial donor cells (Figure 5), we observed significantly higher proportions of LT-HSCs, ST-HSCs and MPPs in the calvaria than in the tibias of mice that received P14 and P21 donor cells.

When we analyzed the levels of the stem cell proliferation and retention factor CXCL12 by ELISA (Figure 5D), we observed significantly higher levels in the calvarium compared to the tibia. CXCL12 is a critical regulator of HSC homing in the bone marrow [21]. These results suggest that irrespective of the source of the hematopoietic cells, the calvarial marrow provides a better microenvironment for the survival and engraftment of HSPCs.

Differential recruitment of hematopoietic cells from the calvarium and tibia into the retina following acute ischemic injury

A major role of the bone marrow is to mobilize and release myeloid inflammatory and vascular reparative cells to injured tissues. We investigated whether there were any differences in the type and proportion of myeloid cells contributed by the calvarium compared to tibia following retinal injury. We differentially labelled the two bone marrow compartments by photoconversion using different experimental groups (Figure 6A). To accomplish this, the calvarium or tibia of separate KIKGR mice were exposed to 405nm laser to photoconvert the green cells of the respective bone marrow cells to red (Figure 6B,C). The mice then underwent I/R injury to the retina.

At baseline (without retinal injury), we observed that the photoconversion rates of total CD45+ cells, as well as the myeloid cells of interest (neutrophils, monocytes and MACs) were significantly higher in the calvarium than the tibia (Figure 7). This was not

surprising as the calvarium is flatter, thinner and wider, offering increased surface area for exposure to laser than the tibia. Also, the photoconversion rate of each cell type was different, presumably due to the differences in their relative abundances in the marrow and relative locations in the marrow. To account for these differences in the experimental groups, the proportion of photoconverted cells was normalized to the baseline photoconversion rate of each cell type at the photoconversion site. When we subjected mice to acute I/R injury, we observed that the most robust response was from MACs irrespective of the photoconverted site (Figure 8A,B). Interestingly, the proportion of neutrophils and MACs but not monocytes from the calvarium were significantly higher than that of the tibia (Figure 8C,D,E).

Myeloid cell composition of the calvarium versus tibia

We asked whether the observed migration heterogeneity to the injured retina could be attributed to differences in the composition of the myeloid cells in the different bone marrow sites. To investigate this, we prepared single cell suspensions of the calvaria and tibial marrows of WT mice and analyzed them by flow cytometry. As shown in Figure 8F, G and H, we observed no significant differences in the proportion of neutrophils and MACs in the calvarium versus the tibia. However, the proportion of monocytes in the tibia was significantly higher than that of the calvarium.

DISCUSSION

The key findings of our study include that in early postnatal development, the calvarium shows a higher proportion of HSPCs and a lower proportion of mature hematopoietic cells compared to the long bones. At time points when the bone marrow dominates hematopoiesis (P14-21), we observed that hematopoietic cells of long bone origin repopulate the peripheral blood of lethally irradiated hosts faster than cells of calvarium origin. These findings indicate a delayed differentiation potential of the calvaria marrow during development. However, the calvaria marrow showed increased engraftment and survival of hematopoietic stem cells and progenitors. This was supported by increased levels of stem cell engraftment and higher expression of the retention factor CXCL12 in the calvaria marrow compared to the long bones. In addition, the calvaria marrow contributed more neutrophils and MACs migrating into the retina following acute ischemic injury than the long bones (tibia).

The development of definitive hematopoiesis begins early in the developing embryo, around E10 in the AGN after primitive hematopoiesis in the embryonic yolk sac [61-63]. From the AGN, hematopoietic stem cells are seeded into other organs such as the spleen, fetal liver and then the bone marrow shortly before birth [8, 64, 65]. Development of both the long bones and flat bones begin prenatally [55-59], and these regions begin to support hematopoiesis when osteoblasts begin to calcify the bones to provide a microenvironment conducive for homing of circulating HSCs into the marrow [66, 67].

However, while the field has made progress in understanding the development of hematopoiesis in the long bones [66, 68, 69], very little is known about the calvarium. By

flow cytometry, we observed that early on in postnatal development (at P7, when the fetal liver still dominates hematopoiesis), the proportion of HSPCs in the calvarium was higher than that of the long bones while the proportion of mature cells was less (Figure 1). At P14 when fetal hematopoiesis has just been shut off due to loss key regulators such as erythropoietin [70], hematopoiesis in the calvarium increased, occupying the marrow with more mature cells (Figure 2). At P21 when the bone marrow is the primary hematopoietic organ, the proportion of HSPCs and mature cells in the calvarium and long bones were not different. Thus, our data uncovered an unsurprisingly gradual increase in hematopoiesis in different bone marrow compartments soon after birth, where the expansion of hematopoiesis in the calvarium trails that of the long bones until at most P21. It has been shown that during embryogenesis, the calvarium appears later, around E12.5, than the long bones around E9 [55, 56]. Although calcification/mineralization, which is required for successful HSC homing and proliferation in the marrow, has been reported to begin around the same time (E14-16 [56, 71]) in both tissues, by E17.5 the entire long bone is calcified but not the calvarium [55, 56], suggesting a relative delay in preparation of the various calvarial segments for HSC function. Our data supports that the relative delay in maturation of the calvarium compared to the long bones is associated with a concomitant delay in hematopoiesis.

Next, we assessed the function of hematopoietic cells from the two locations using the competitive repopulation assay. The competitive repopulation assay, in which isolated bone marrow cells are transplanted into lethally irradiated hosts to replace the hosts hematopoietic cells, is considered the gold standard for evaluating the self-renewal and multilineage capability of stem and progenitor cells in vivo [2, 72, 73]. We employed

this assay to assess the ability of hematopoietic cells from the calvarium vs. long bones to repopulate the blood of irradiated hosts and the engraftment of HSPCs in the calvarium vs. long bones. At all three time points studied, we observed that cells of tibial origin repopulate the blood more rapidly than those of calvaria origin (Figure 3). Hematopoietic cells of different origins have different reconstitution potential [74]. For example, several studies have reported that the fetal liver HSCs have a more rapid reconstitution potential than that of the adult bone marrow [75-77]. A major drawback in these studies is that the donor cells were isolated from sites at different time points. Our data shows that at the same time points when the bone marrow dominates hematopoiesis in the body, cells from different bone marrow compartments exhibit different rates of blood reconstitution.

When we assessed the hosts' bone marrows 16 weeks post transplantation, a time point accepted as stable reconstitution, there was no difference in bone marrow stem and progenitors in hosts that received donor calvarial and long bone cells from P7 pups. This is not surprising because at P7, both bone marrows are still relatively immature. Immature bone marrow stroma does not possess the optimal conditions required for optimal engraftment, maturation and differentiation of stem cells [78]. Thus, cells of both long bone and calvaria origin from P7 pups are similar and not optimally primed for engraftment. However, analysis of bone marrow of hosts transplanted with cells from P14 and P21 pups showed that, irrespective of the source of cells, the proportion of LT-HSCs, ST-HSCs and MPPs in the calvarium was higher that of the long bones (tibia) (Figures 4 and 5). To ascertain a potential mechanism by which HSPCs survive and engraft better in the calvarium, we analyzed the levels of CXCL12 in the bone marrows by ELISA. CXCL12 is an important regulator of hematopoietic stem cell engraftment and facilitates

HSC retention at hematopoietic sites [66, 79, 80]. It binds to its receptor, CXCR4, expressed on HSCs. Activation of the CXCL12/CXCR4 signaling promotes HSC homing and repopulation in the marrow and the maintenance of HSC quiescence [81, 82]. We observed that CXCL12 was higher in the calvarium compared to the long bones. Taken together, our data suggests that the calvarium offers a better microenvironment for engraftment and survival of hematopoietic stem and progenitor cells by promoting increased expression of CXCL12.

In addition to replenishing peripheral blood cells, the bone marrow responds to stress and injury by releasing inflammatory and reparatory cells that migrate to sites of injury [33, 83-85]. The injured retina, like all tissues, is invaded by bone marrow-derived cells following injury and hematopoietic cells contribute to the repair of the damage and reduce progression [86-88]. Bone marrow exhibits functional heterogeneity, in which different compartments or sites contribute different proportions of inflammatory cells into injured tissues [38]. We investigated whether this functional heterogeneity is exhibited in response to acute retinal injury. In vivo bone marrow labelling by photoconversion was followed by retinal ischemia-reperfusion injury. IR injury is a pathophysiologic phenomenon whereby tissue damage by hypoxia is exacerbated by reperfusion injury [23]. This injury model recapitulates neuronal cell loss mediated by severe vascular injury and inflammation [22, 89, 90]. We observed that after ischemic injury, the most robust cellular response from either the calvarium or tibia was by MACs. This was not unexpected as MACs are vascular reparative cells that provide paracrine support to facilitate repair in injured tissues. Interestingly, we observed that the calvaria marrow contributed more neutrophils and MACs. Herrisson et. al. reported a similar finding with

neutrophils in the brain when the mice were subjected to acute ischemic stroke [38]. Whereas MACs are known to be vascular reparative and beneficial, the role of neutrophils in tissue injury has been reported to be both beneficial and destructive [24, 91]. However, as first responders to tissue injury, neutrophils promote neuronal survival by expressing important paracrine factors [92]. Thus, our data supports that the calvaria marrow contributes more of the early vascular and neuroprotective cell types from the bone marrow following acute injury.

We observed no significant differences in the proportion of neutrophils and MACs between the calvarial marrow and the tibia, except for monocytes which was higher in the tibia. Thus, the proportion of cells in the marrows did not account for the heterogeneity in recruitment of bone marrow cells into the injured retina. However, the calvaria marrow communicates with the central nervous system through the cerebrospinal fluid (CSF). CSF directly accesses the calvaria marrow through the perivascular space bordering the vasculature that enter the calvarium and bacteria take advantage of this route to infect hematopoietic niches following meningitis [93]. The CSF within the subarachnoid space is contiguous with and surrounds the optic nerve head at the back of the eye [94]. It is known that injury signals such as tumor necrosis factor alpha and interferons are essential cytokines of inflammation that affect hematopoiesis [95, 96]. Thus, it is plausible that injury signals from the retina travel through the CSF at the back of the eye to the calvarium and elicit a calvarial marrow response faster than the tibial marrow which lacks direct input from the CSF. This could lead to the initial high contribution of cells from the calvarium we observed. Although studies have reported entry of bone marrow-derived cells into the retina via the optic nerve head [16], further

studies will look to explore evidence of this potential route of communication between the retina and the calvaria marrow through the CSF.

CONCLUSION

Our study has provided insight into differences in hematopoiesis in the calvarium and long bone compartments. The expansion of hematopoietic cells during development is delayed in the calvaria marrow compared to the long bones until P21. Cells of calvarial origin do not repopulate blood as quickly as those of long bone origin in competitive repopulation studies. However, the calvaria marrow promotes improved survival of HSPCs by increased expression of stem cell factors such as CXCL12. Our data also supports that the calvarium contributes more neutrophils and more vascular reparative cells to the retina following acute injury. Further investigation is required to determine if this is mediated by faster communication between the retina and the calvarial marrow through CSF. Our study supports that even though the bone marrow is the primary hematopoietic organ in the adult state, hematopoiesis in different bone marrow compartments is functionally different. It also sheds light on the previously underappreciated importance of the calvaria bone marrow as a unique source of reparative cells for the retina and survival of HSPCs.

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REFERENCES

1. Cumano, A. and I. Godin, Ontogeny of the hematopoietic system. Annual review of immunology, 2007. 25(1): p. 745-785.

2. Warren, L.A. and D.J. Rossi, Stem cells and aging in the hematopoietic system. Mechanisms of ageing and development, 2009. 130(1-2): p. 46-53.

3. Jagannathan-Bogdan, M. and L.I. Zon, Hematopoiesis. Development, 2013. 140(12): p. 2463-2467.

4. Abkowitz, J.L., et al., Behavior of hematopoietic stem cells in a large animal. Proceedings of the National Academy of Sciences, 1995. 92(6): p. 2031-2035.

5. Hannoush, E.J., et al., Impact of enhanced mobilization of bone marrow derived cells to site of injury. Journal of Trauma and Acute Care Surgery, 2011. 71(2): p. 283-291.

6. Harris, J.R., et al., Bone marrow–derived cells home to and regenerate retinal pigment epithelium after injury. Investigative ophthalmology & visual science, 2006. 47(5): p. 2108-2113.

7. Caballero, S., et al., Bone Marrow–Derived Cell Recruitment to the Neurosensory Retina and Retinal Pigment Epithelial Cell Layer Following Subthreshold Retinal Phototherapy. Investigative ophthalmology & visual science, 2017. 58(12): p. 5164-5176.

8. Rieger, M.A. and T. Schroeder, Hematopoiesis. Cold Spring Harbor perspectives in biology, 2012. 4(12): p. a008250.

9. Yoder, M., Embryonic hematopoiesis in mice and humans. Acta Pædiatrica, 2002. 91: p. 5-8.

10. Galloway, J.L. and L.I. Zon, 3 ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. 2003.

11. Morrison, S.J. and D.T. Scadden, The bone marrow niche for haematopoietic stem cells. Nature, 2014. 505(7483): p. 327-334.

12. Papa, L., M. Djedaini, and R. Hoffman, Mitochondrial role in stemness and differentiation of hematopoietic stem cells. Stem cells international, 2019. 2019.

13. Bryder, D., D.J. Rossi, and I.L. Weissman, Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. The American journal of pathology, 2006. 169(2): p. 338-346.

14. Orkin, S.H., Development of the hematopoietic system. Current opinion in genetics & development, 1996. 6(5): p. 597-602.

15. Orkin, S.H., Hematopoiesis: how does it happen? Current opinion in cell biology, 1995. 7(6): p. 870-877.

16. Kaneko, H., et al., Characteristics of bone marrow–derived microglia in the normal and injured retina. Investigative ophthalmology & visual science, 2008. 49(9): p. 4162-4168.

17. London, A., et al., Neuroprotection and progenitor cell renewal in the injured adult murine retina requires healing monocyte-derived macrophages. Journal of Experimental Medicine, 2011. 208(1): p. 23-39.

18. O'Koren, E., R. Mathew, and D. Saban, Fate mapping reveals that microglia and recruited monocyte-derived macrophages are definitively distinguishable by phenotype in the retina. Scientific reports, 2016. 6(1): p. 1-12.

19. Chakravarthy, H., et al., Imbalances in mobilization and activation of pro-inflammatory and vascular reparative bone marrow-derived cells in diabetic retinopathy. PloS one, 2016. 11(1): p. e0146829.

20. Sasahara, M., et al., Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration. The American journal of pathology, 2008. 172(6): p. 1693-1703.

21. Purcell, B.P., et al., Synergistic effects of SDF-1α chemokine and hyaluronic acid release from degradable hydrogels on directing bone marrow derived cell homing to the myocardium. Biomaterials, 2012. 33(31): p. 7849-7857.

22. Kielar, M.L., et al., Maladaptive role of IL-6 in ischemic acute renal failure. Journal of the American Society of Nephrology, 2005. 16(11): p. 3315-3325.

23. Tsung, A., et al., Hepatic ischemia/reperfusion injury involves functional TLR4 signaling in nonparenchymal cells. The Journal of Immunology, 2005. 175(11): p. 7661-7668.

24. Segel, G.B., M.W. Halterman, and M.A. Lichtman, The paradox of the neutrophil's role in tissue injury. Journal of leukocyte biology, 2011. 89(3): p. 359-372.

25. Kim, S.Y., et al., Neutrophils are associated with capillary closure in spontaneously diabetic monkey retinas. Diabetes, 2005. 54(5): p. 1534-1542.

26. Liu, Y., et al., Reversible retinal vessel closure from VEGF-induced leukocyte plugging. JCI insight, 2017. 2(18).

27. Joussen, A.M., et al., Leukocyte-mediated endothelial cell injury and death in the diabetic retina. The American journal of pathology, 2001. 158(1): p. 147-152.

28. Ghosh, S., et al., Neutrophils homing into the retina trigger pathology in early age-related macular degeneration. Communications biology, 2019. 2(1): p. 1-17.

29. Paschalis, E.I., et al., The role of microglia and peripheral monocytes in retinal damage after corneal chemical injury. The American journal of pathology, 2018. 188(7): p. 1580-1596.

30. Benhar, I., et al., The retinal pigment epithelium as a gateway for monocyte trafficking into the eye. The EMBO journal, 2016. 35(11): p. 1219-1235.

31. Asare-Bediako, B., et al., Hematopoietic Cells Influence Vascular Development in the Retina. Cells, 2022. 11(20): p. 3207.

32. Paschalis, E.I., et al., Permanent neuroglial remodeling of the retina following infiltration of CSF1R inhibition-resistant peripheral monocytes. Proceedings of the National Academy of Sciences, 2018. 115(48): p. E11359-E11368.

33. Duan, Y., et al., Bone marrow-derived cells restore functional integrity of the gut epithelial and vascular barriers in a model of diabetes and ACE2 deficiency. Circulation research, 2019. 125(11): p. 969-988.

34. Medina, R.J., et al., Endothelial progenitors: a consensus statement on nomenclature. Stem cells translational medicine, 2017. 6(5): p. 1316-1320.

35. Urbich, C., et al., Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. Journal of molecular and cellular cardiology, 2005. 39(5): p. 733-742.

36. Medina, R.J., et al., Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. Molecular medicine, 2011. 17(9): p. 1045-1055.

37. Wilson, A. and A. Trumpp, Bone-marrow haematopoietic-stem-cell niches. Nature Reviews Immunology, 2006. 6(2): p. 93-106.

38. Herisson, F., et al., Direct vascular channels connect skull bone marrow and the brain surface enabling myeloid cell migration. Nature neuroscience, 2018. 21(9): p. 1209-1217.

39. Lo Celso, C., C.P. Lin, and D.T. Scadden, In vivo imaging of transplanted hematopoietic stem and progenitor cells in mouse calvarium bone marrow. Nature protocols, 2011. 6(1): p. 1-14.

40. Mills III, W.A., M.A. Coburn, and U.B. Eyo, The emergence of the calvarial hematopoietic niche in health and disease. Immunological Reviews, 2022. 311(1): p. 26-38.

41. Mazo, I.B., et al., Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. The Journal of experimental medicine, 1998. 188(3): p. 465-474.

42. Lo Celso, C., et al., Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature, 2009. 457(7225): p. 92-96.

43. Sipkins, D.A., et al., In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature, 2005. 435(7044): p. 969-973.

44. Knospe, W.H., et al., Origin and recovery of colony-forming units in locally curetted bone marrow of mice. Blood, 1972. 39(3): p. 331-340.

45. Knospe, W.H., et al., Stimulation of hematopoiesis by femoral marrow curettage in sublethally irradiated mice. Blood, 1973. 41(4): p. 519-527.

46. Trottier, M.D., et al., Enhancement of hematopoiesis and lymphopoiesis in diet-induced obese mice. Proceedings of the National Academy of Sciences, 2012. 109(20): p. 7622-7629.

47. Markina, E.A., et al., Short-Term Reloading After Prolonged Unloading Ensures Restoration of Stromal but Not Hematopoietic Precursor Activity in Tibia Bone Marrow of C57Bl/6N Mice. Stem Cells and Development, 2021. 30(24): p. 1228-1240.

48. Kim, S., et al., Extended time-lapse in vivo imaging of tibia bone marrow to visualize dynamic hematopoietic stem cell engraftment. Leukemia, 2017. 31(7): p. 1582-1592.

49. Grüneboom, A., et al., A network of trans-cortical capillaries as mainstay for blood circulation in long bones. Nature Metabolism, 2019. 1(2): p. 236-250.

50. Jafri, S., et al., A sex-specific reconstitution bias in the competitive CD45. 1/CD45. 2 congenic bone marrow transplant model. Scientific reports, 2017. 7(1): p. 1-8.

51. Mahajan, M., et al., A quantitative assessment of the content of hematopoietic stem cells in mouse and human endosteal-bone marrow: a simple and rapid method for the isolation of mouse central bone marrow. BMC Hematol. 2015; 15: 9.

52. Park, S.S., et al., Long-Term Effects of Intravitreal Injection of GMP-Grade Bone-Marrow–Derived CD34+ Cells in NOD-SCID Mice with Acute Ischemia-Reperfusion Injury. Investigative ophthalmology & visual science, 2012. 53(2): p. 986-994.

53. Caballero, S., et al., Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. Diabetes, 2007. 56(4): p. 960-967.

54. Luis, T., N. Killmann, and F. Staal, Signal transduction pathways regulating hematopoietic stem cell biology: introduction to a series of Spotlight Reviews. Leukemia, 2012. 26(1): p. 86-90.

55. Jiang, X., et al., Tissue origins and interactions in the mammalian skull vault. Developmental biology, 2002. 241(1): p. 106-116.

56. Ishii, M., et al., The development of the calvarial bones and sutures and the pathophysiology of craniosynostosis. Current topics in developmental biology, 2015. 115: p. 131-156.

57. Hankenson, K.D., et al., Mice with a disruption of the thrombospondin 3 gene differ in geometric and biomechanical properties of bone and have accelerated development of the femoral head. Molecular and cellular biology, 2005. 25(13): p. 5599-5606.

58. Greene, R. and D. Kochhar, Limb development in mouse embryos: protection against teratogenic effects of 6-diazo-5 oxo-L-norleucine (DON) in vivo and in vitro. 1975.

59. Sekine, K., et al., Fgf10 is essential for limb and lung formation. Nature genetics, 1999. 21(1): p. 138-141.

60. Orkin, S.H. and L.I. Zon, Hematopoiesis: an evolving paradigm for stem cell biology. Cell, 2008. 132(4): p. 631-644.

61. Taoudi, S., et al., Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+ CD45+ pre-definitive HSCs. Cell stem cell, 2008. 3(1): p. 99-108.

62. Kumaravelu, P., et al., Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. 2002.

63. Medvinsky, A.L., et al., An early pre-liver intraembryonic source of CFU-S in the developing mouse. Nature, 1993. 364(6432): p. 64-67.

64. Mikkola, H.K. and S.H. Orkin, The journey of developing hematopoietic stem cells. 2006.

65. Samokhvalov, I.M., N.I. Samokhvalova, and S.-i. Nishikawa, Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. Nature, 2007. 446(7139): p. 1056-1061.

66. Christensen, J.L., et al., Circulation and chemotaxis of fetal hematopoietic stem cells. PLoS biology, 2004. 2(3): p. e75.

67. Adams, G.B. and D.T. Scadden, The hematopoietic stem cell in its place. Nature immunology, 2006. 7(4): p. 333-337.

68. Coşkun, S., et al., Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. Cell reports, 2014. 9(2): p. 581-590.

69. Hall, T.D., et al., Murine fetal bone marrow does not support functional hematopoietic stem and progenitor cells until birth. Nature Communications, 2022. 13(1): p. 1-18.

70. Suzuki, N., et al., Specific contribution of the erythropoietin gene 3′ enhancer to hepatic erythropoiesis after late embryonic stages. Molecular and cellular biology, 2011. 31(18): p. 3896-3905.

71. Neubert, D., H.-J. Merker, and S. Tapken, Comparative studies on the prenatal development of mouse extremities in vivo and in organ culture. Naunyn-Schmiedeberg's Archives of Pharmacology, 1974. 286(3): p. 251-270.

72. Müller, A.M., et al., Development of hematopoietic stem cell activity in the mouse embryo. Immunity, 1994. 1(4): p. 291-301.

73. Moore, M.A. and D. Metcalf, Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. British journal of haematology, 1970. 18(3): p. 279-296.

74. Kondo, M., et al., Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annual review of immunology, 2003. 21(1): p. 759-806.

75. Jordan, C., et al., Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. Experimental hematology, 1995. 23(9): p. 1011-1015.

76. Rebel, V.I., et al., The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. 1996.

77. Holyoake, T.L., F.E. Nicolini, and C.J. Eaves, Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Experimental hematology, 1999. 27(9): p. 1418-1427.

78. Zanjani, E.D., J.L. Ascensao, and M. Tavassoli, Liver-derived fetal hematopoietic stem cells selectively and preferentially home to the fetal bone marrow. 1993.

79. Lewis, K., M. Yoshimoto, and T. Takebe, Fetal liver hematopoiesis: from development to delivery. Stem Cell Research & Therapy, 2021. 12(1): p. 1-8.

80. Suárez-Álvarez, B., A. López-Vázquez, and C. López-Larrea, Mobilization and homing of hematopoietic stem cells. Stem cell transplantation, 2012: p. 152-170.

81. Sugiyama, T., et al., Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity, 2006. 25(6): p. 977-988.

82. Moll, N.M. and R.M. Ransohoff, CXCL12 and CXCR4 in bone marrow physiology. Expert review of hematology, 2010. 3(3): p. 315-322.

83. Zhang, Y., et al., Bone marrow injury induced via oxidative stress in mice by inhalation exposure to formaldehyde. PloS one, 2013. 8(9): p. e74974.

84. Wang, Y., et al., Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. Free Radical Biology and Medicine, 2010. 48(2): p. 348-356.

85. Sengupta, N., et al., The role of adult bone marrow-derived stem cells in choroidal neovascularization. Investigative ophthalmology & visual science, 2003. 44(11): p. 4908-4913.

86. Grant, M.B., et al., Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nature medicine, 2002. 8(6): p. 607-612.

87. Hazra, S., et al., Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model. Diabetologia, 2013. 56(3): p. 644-653.

88. Bhatwadekar, A.D., et al., Hematopoietic stem/progenitor involvement in retinal microvascular repair during diabetes: Implications for bone marrow rejuvenation. Vision research, 2017. 139: p. 211-220.

89. Molitoris, B.A., Transitioning to therapy in ischemic acute renal failure. Journal of the American Society of Nephrology, 2003. 14(1): p. 265-267.

90. Bonventre, J.V. and J.M. Weinberg, Recent advances in the pathophysiology of ischemic acute renal failure. Journal of the American Society of Nephrology, 2003. 14(8): p. 2199-2210.

91. Liu, Y.-W., S. Li, and S.-S. Dai, Neutrophils in traumatic brain injury (TBI): friend or foe? Journal of neuroinflammation, 2018. 15(1): p. 1-18.

92. Kurimoto, T., et al., Neutrophils express oncomodulin and promote optic nerve regeneration. Journal of Neuroscience, 2013. 33(37): p. 14816-14824.

93. Pulous, F.E., et al., Cerebrospinal fluid can exit into the skull bone marrow and instruct cranial hematopoiesis in mice with bacterial meningitis. Nature Neuroscience, 2022. 25(5): p. 567-576.

94. Wostyn, P., et al., A new glaucoma hypothesis: a role of glymphatic system dysfunction. Fluids and Barriers of the CNS, 2015. 12(1): p. 1-6.

95. Dzierzak, E. and A. Bigas, Blood development: hematopoietic stem cell dependence and independence. Cell stem cell, 2018. 22(5): p. 639-651.

96. Schuettpelz, L.G. and D.C. Link, Regulation of hematopoietic stem cell activity by inflammation. Frontiers in immunology, 2013. 4: p. 204.

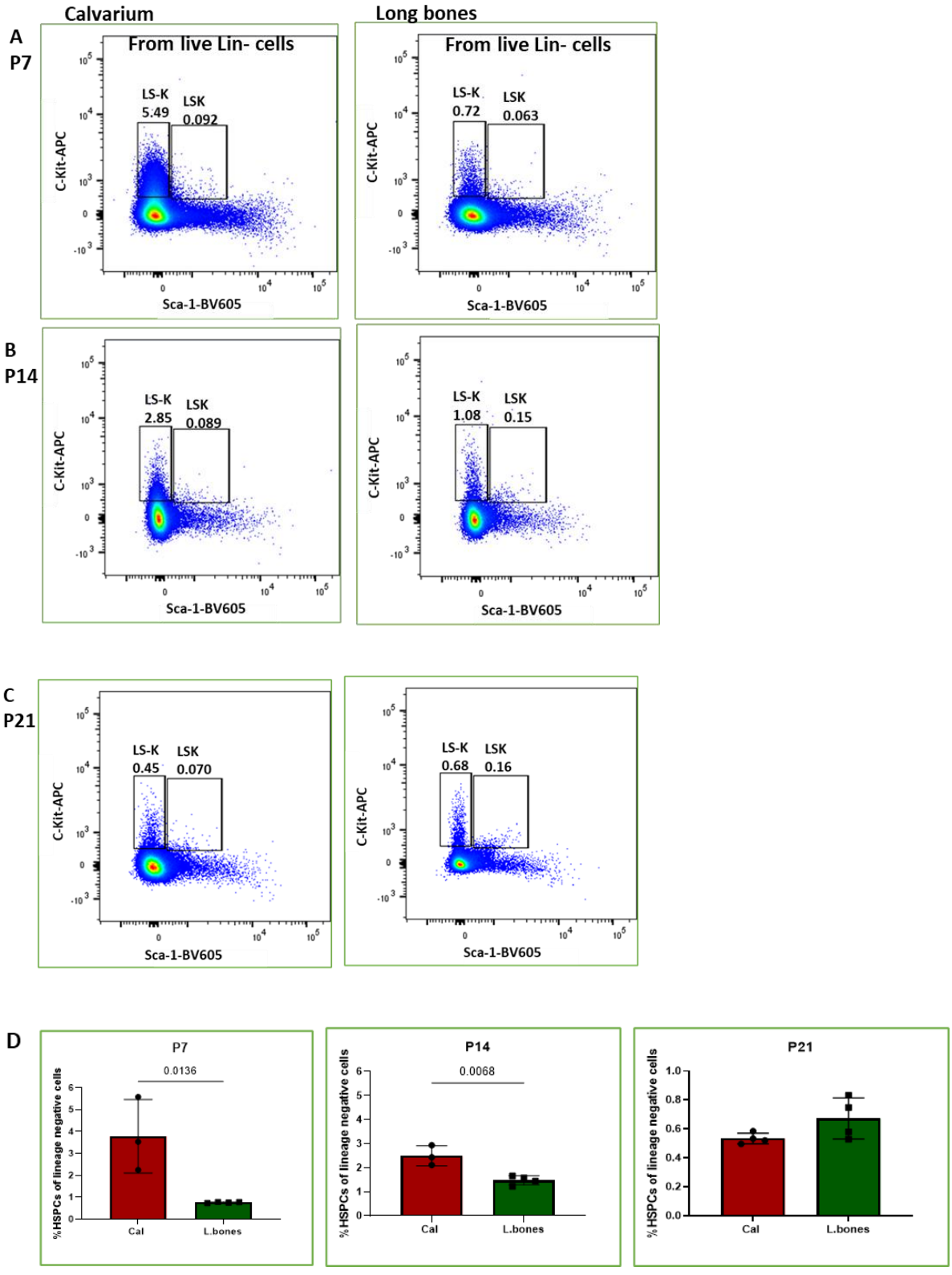
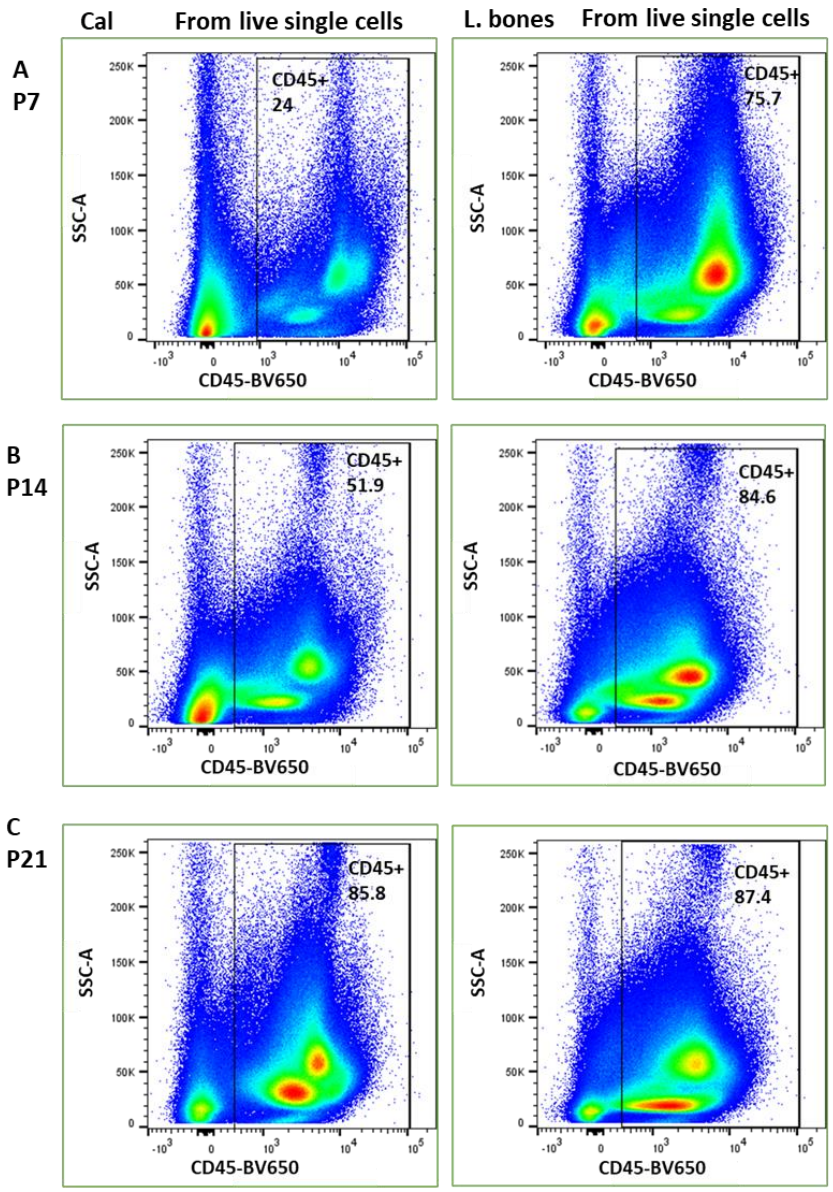

Calvarium
Long bones
A
P7
From live Lin- cells
LS-K
5.49
LSK
0.092
C-Kit-APC
Sca-1-BV605
From live Lin- cells
LS-K
0.72
LSK
0.063
C-Kit-APC
Sca-1-BV605
B
P14
LS-K
2.85
LSK
0.089
C-Kit-APC
Sca-1-BV605
LS-K
1.08
LSK
0.15
C-Kit-APC
Sca-1-BV605
C
P21
LS-K
0.45
LSK
0.070
C-Kit-APC
Sca-1-BV605
LS-K
0.68
LSK
0.16
C-Kit-APC
Sca-1-BV605
D
P7
0.0136
%HSPCs of lineage negative cells
Cal
L.bones
P14
0.0068
%HSPCs of lineage negative cells
Cal
L.bones
P21
%HSPCs of lineage negative cells
Cal
L.bones

Figure 1: Hematopoietic stem and progenitor cells (HSPCs) in the calvarium and long bones during postnatal development. A-C: Representative flow cytometry plots for hematopoietic stem and progenitor cells (LS-K and LSK combined) in the bone marrow. D: Bar graphs showing quantification of HSPCs at the different time points. The proportion of HSPCs was significantly higher in the calvarium than long bones at P7 and P14 but decreased to comparable levels at P21 as hematopoiesis increased in the calvarium over time.


Cal
From live single cells
L. bones
From live single cells
A
P7
B
P14
C
P21
CD45+
24
CD45+
75.7
CD45+
51.9
CD45+
84.6
CD45+
85.8
CD45+
87.4
SSC-A
CD45-BV650

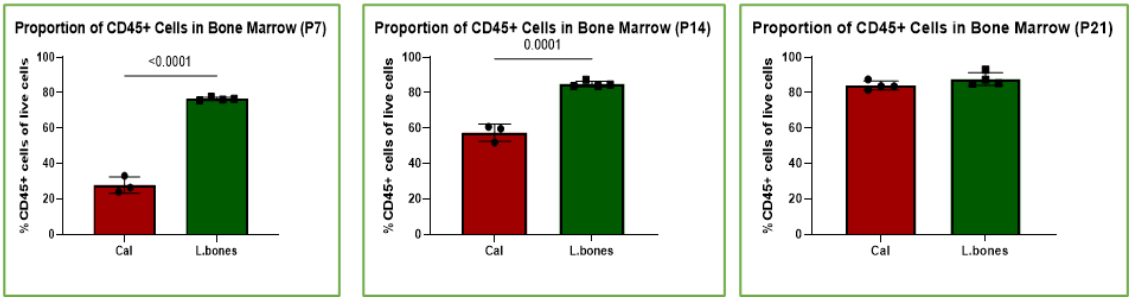
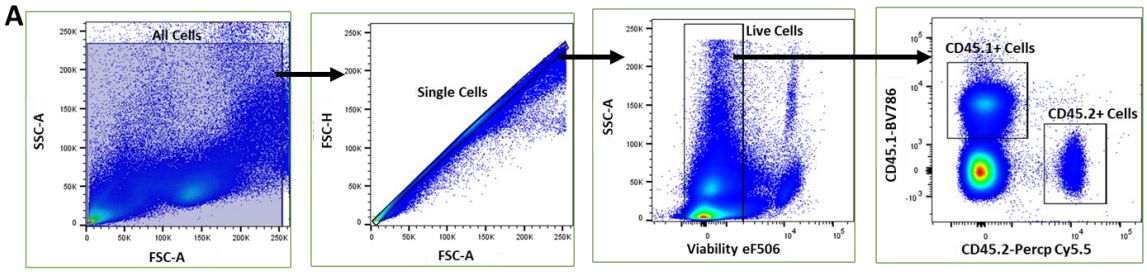
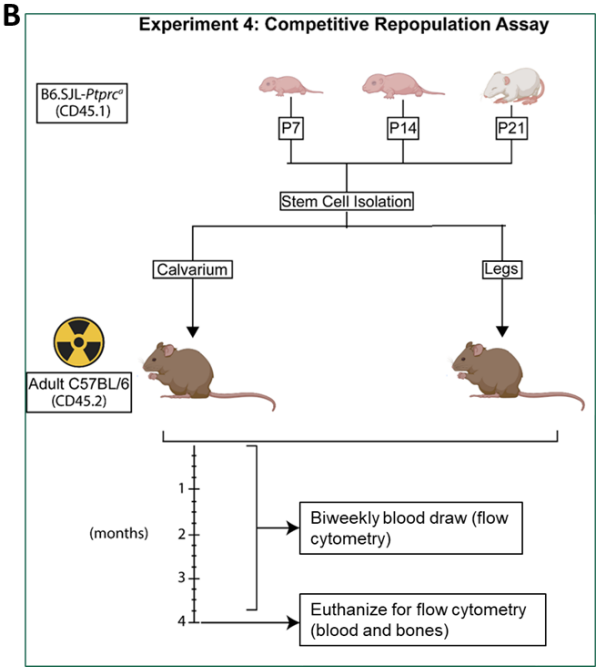
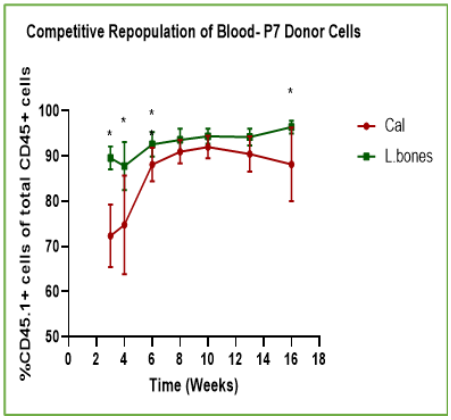
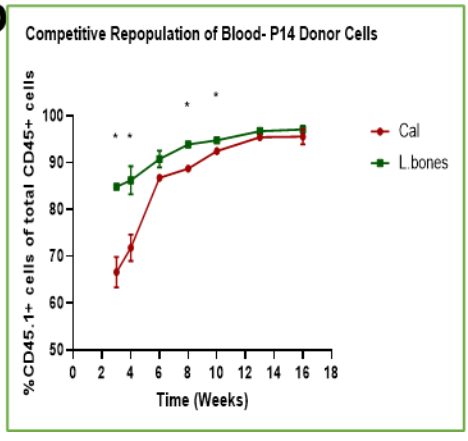

D
Proportion of CD45+ Cells in Bone Marrow (P7)
<0.0001
Proportion of CD45+ Cells in Bone Marrow (P14)
0.0001
Proportion of CD45+ Cells in Bone Marrow (P21)
% CD45+ cells of live cells
Cal
L.bones

Figure 2: Mature hematopoietic cells (CD45+) in the calvarium and long bones during postnatal development. A-C: Representative flow cytometry plots for CD45+ cells in the bone marrow. D: Bar graphs showing quantification of CD45+ cells at the different time points. The proportion of CD45+ cells was significantly lower in the calvarium than long bones at P7 and P14 but increased to comparable levels at P21 as hematopoiesis increased in the calvarium over time.


A
All Cells
SSC-A
FSC-A
Single Cells
FSC-H
FSC-A
Live Cells
SSC-A
Viability eF506
CD45.1+ Cells
CD45.2+ Cells
CD45.1-BV786
CD45.2-Percp Cy5.5


B
Experiment 4: Competitive Repopulation Assay
B6.SJL-Ptprc^a (CD45.1)
P7
P14
P21
Stem Cell Isolation
Calvarium
Legs
Adult C57BL/6 (CD45.2)
(months)
1
2
3
4
Biweekly blood draw (flow cytometry)
Euthanize for flow cytometry (blood and bones)


C
Competitive Repopulation of Blood- P7 Donor Cells
%CD45.1+ cells of total CD45+ cells
Time (Weeks)
Cal
L.bones


D
Competitive Repopulation of Blood- P14 Donor Cells
%CD45.1+ cells of total CD45+ cells
Time (Weeks)
Cal
L.bones

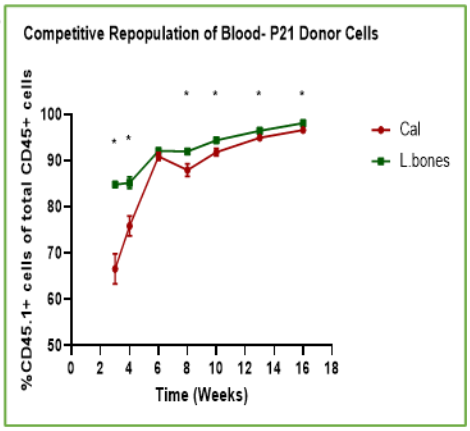
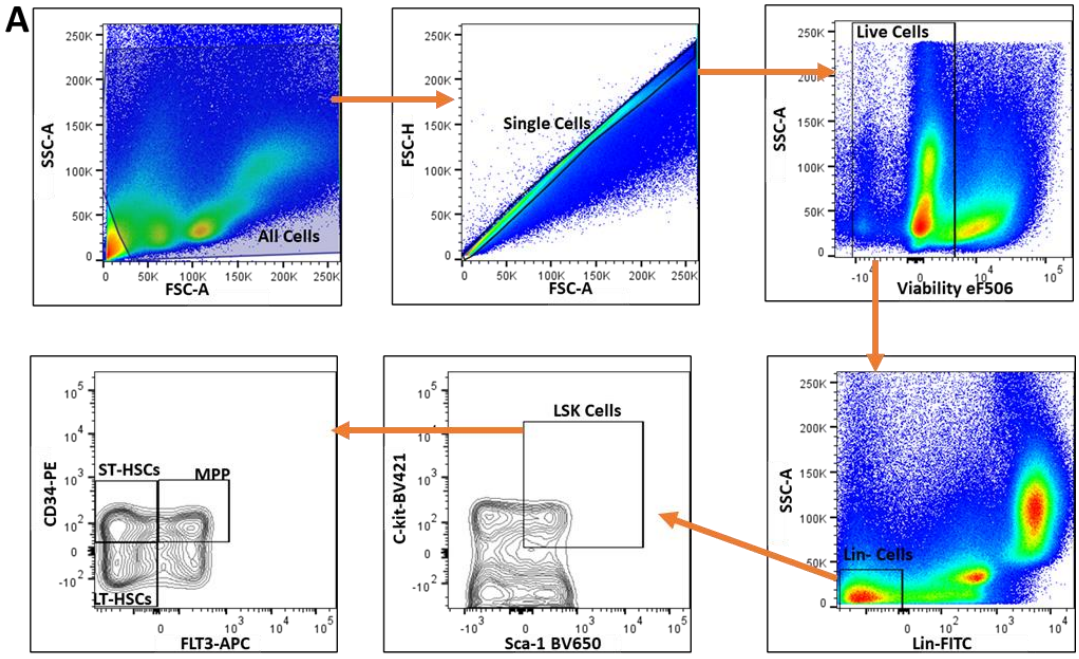

E
Competitive Repopulation of Blood- P21 Donor Cells
%CD45.1+ cells of total CD45+ cells
Time (Weeks)
Cal
L.bones

Figure 3: Assessment of hematopoietic cell function in calvarium vs. long bones by competitive repopulation assay. A: Representative flow plots showing flow cytometric gating strategy for CD45.1+ donor cells and CD45.2+ host cells in the blood. B: Experimental design for the competitive repopulation assay. Bone marrow-derived cells were isolated from the calvarium and long bones of P7, P14 and P21 CD45.1+ donors and transplanted into lethally irradiated CD45.2+ hosts. Mice were bled biweekly and euthanized 4 months post transplantation for flow cytometry. C-E: Line graphs showing the proportion of donor cells reconstituting the blood of hosts.


A
SSC-A
FSC-A
All Cells
FSC-H
Single Cells
Live Cells
Viability eF506
Lin- Cells
Lin-FITC
LSK Cells
Sca-1 BV650
C-kit-BV421
ST-HSCs
MPP
LT-HSCs
CD34-PE
FLT3-APC

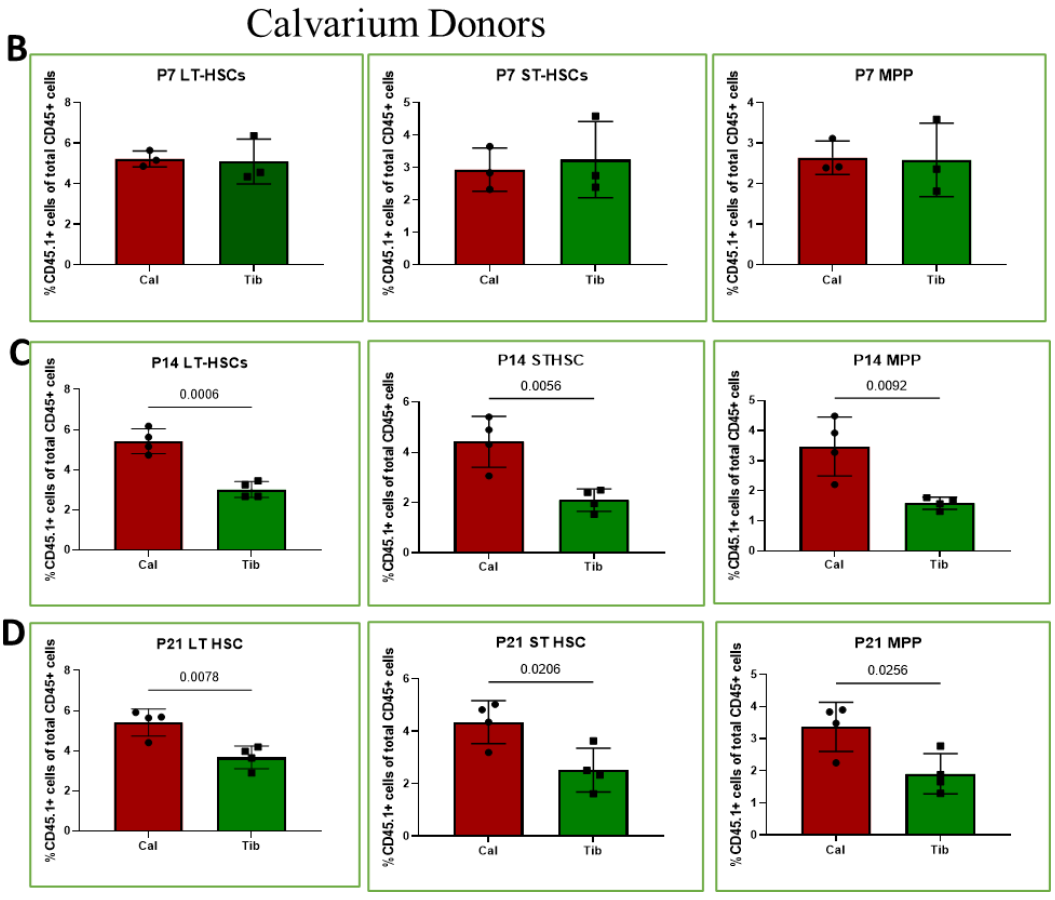

Calvarium Donors
B
P7 LT-HSCs
P7 ST-HSCs
P7 MPP
% CD45.1+ cells of total CD45+ cells
Cal
Tib
C
P14 LT-HSCs
P14 STHSC
P14 MPP
0.0006
0.0056
0.0092
D
P21 LT HSC
P21 ST HSC
P21 MPP
0.0078
0.0206
0.0256

Figure 4: Engraftment of hematopoietic stem and progenitor cells in the bone marrow 4 months post transplantation. A: Representative plots showing flow cytometric gating strategy for evaluation of LT-HSCs, ST-HSCs and MPPs in the bone marrow. Mice were euthanized 16 weeks post transplantation and the proportion of hematopoietic stem and progenitors in the long bones (tibia) and flat bones (calvarium) assessed by flow cytometry. B-D: Bar charts showing quantification of LT-HSCs, ST-HSCs and MPP in bone marrows of hosts that received calvarial donor cells at P7, P14 and P21 respectively.

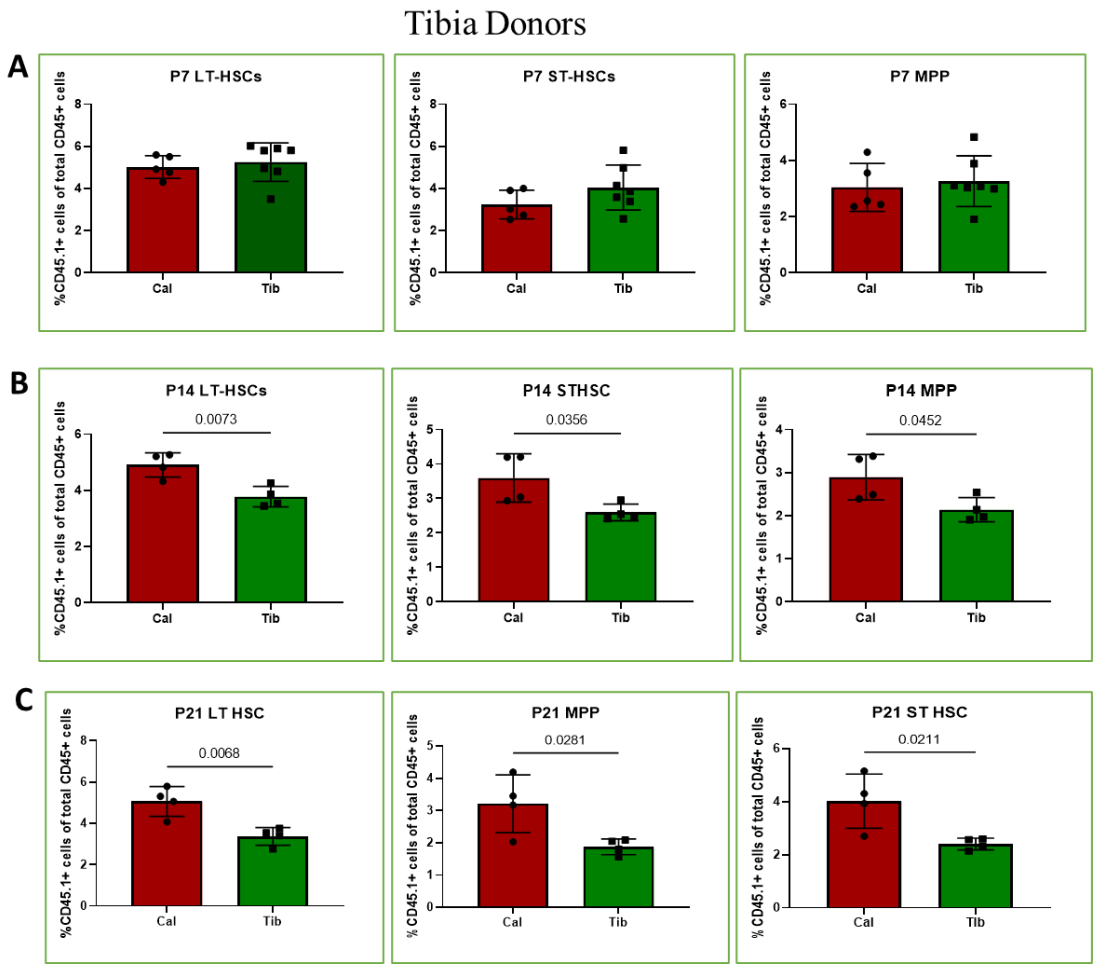


Figure 5: Engraftment of hematopoietic stem and progenitor cells in the bone marrow 4 months post transplantation. A-C: Bar charts showing quantification of LT-HSCs, ST-HSCs and MPP in bone marrows of hosts that received tibia donor cells at P7, P14 and P21 respectively. D: Quantification of CXCL12 levels in the calvarium and tibia (prior to irradiation and transplantation), assessed by ELISA showing significantly higher levels of CXCL12 in the calvarium compared to tibia.

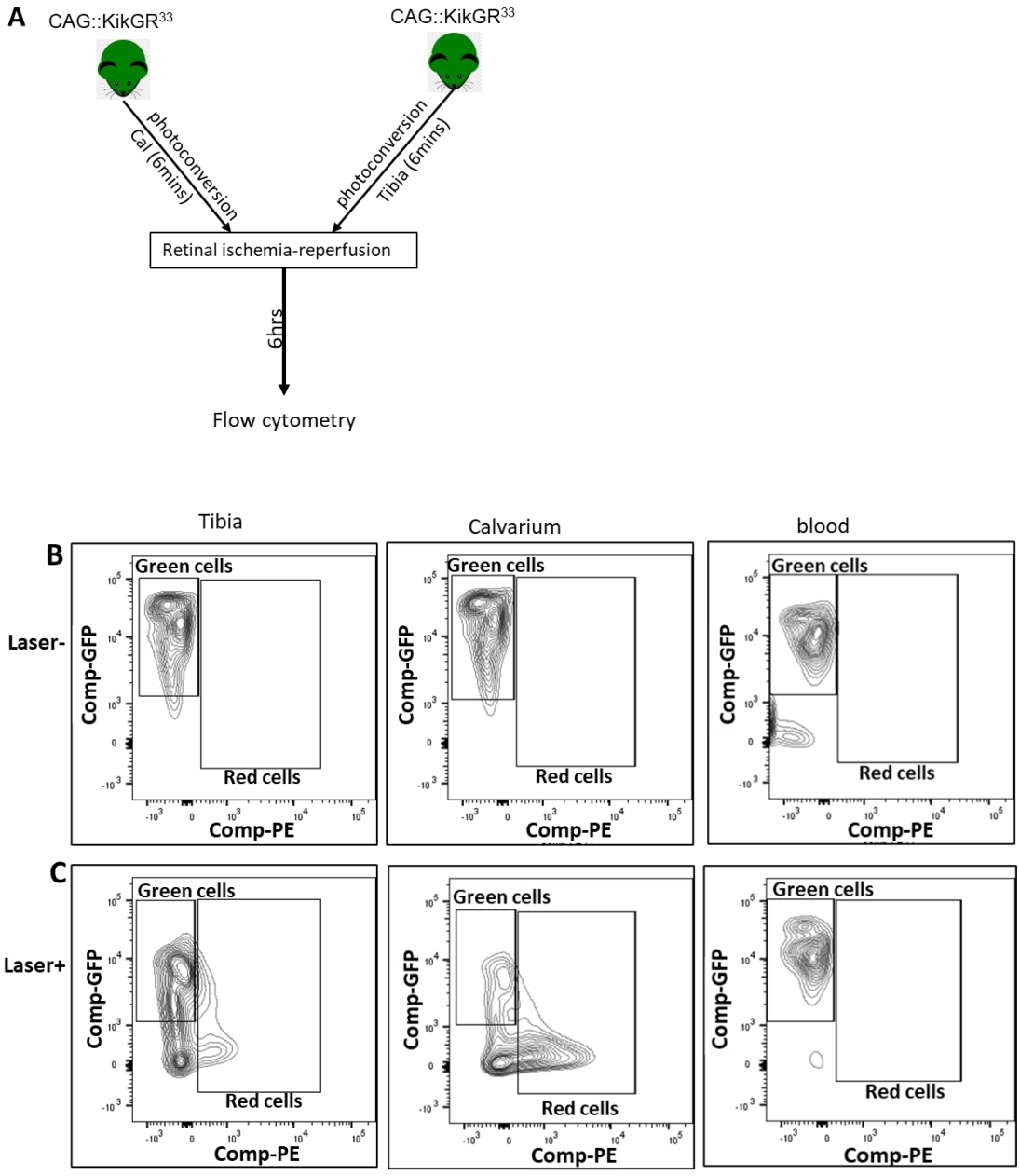
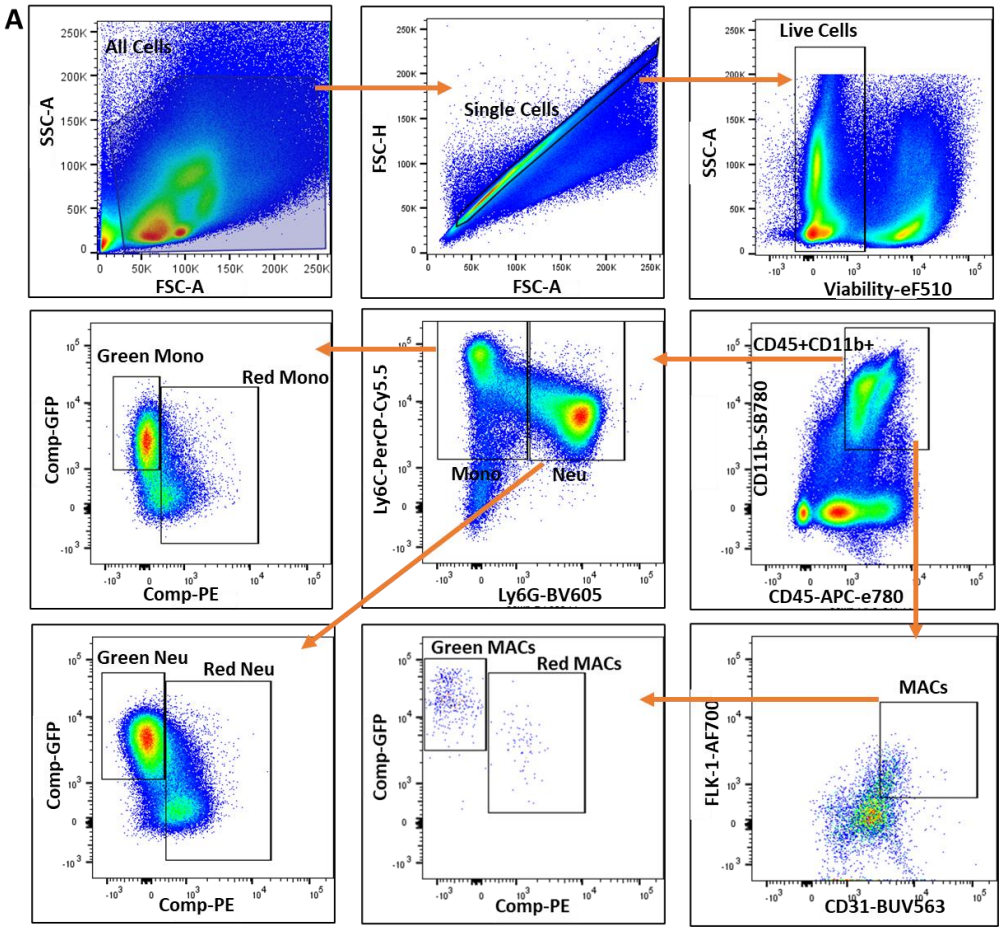

A
CAG::KikGR33
CAG::KikGR33
photoconversion
Cal (6mins)
photoconversion
Tibia (6mins)
Retinal ischemia-reperfusion
6hrs
Flow cytometry
Tibia
Calvarium
blood
B
Laser-
C
Laser+
Green cells
Red cells
Comp-GFP
Comp-PE

Figure 6: In vivo bone marrow cell labelling by photoconverting calvarial and tibial marrow of KIKGR mice for BM cell tracking. A: Experimental design for tracking recruitment of BM cells into the injured retina. Either the tibias or calvaria of KIKGR mice were photoconverted by laser exposure, followed by retinal ischemia reperfusion and then flow cytometry 6 hours later. B,C: Representative plots showing proportions of live photoconverted (PE:red) CD45+ cells in the tibia, calvarium and blood of control uninjured mice before (B) and immediately after (C) photoconversion.



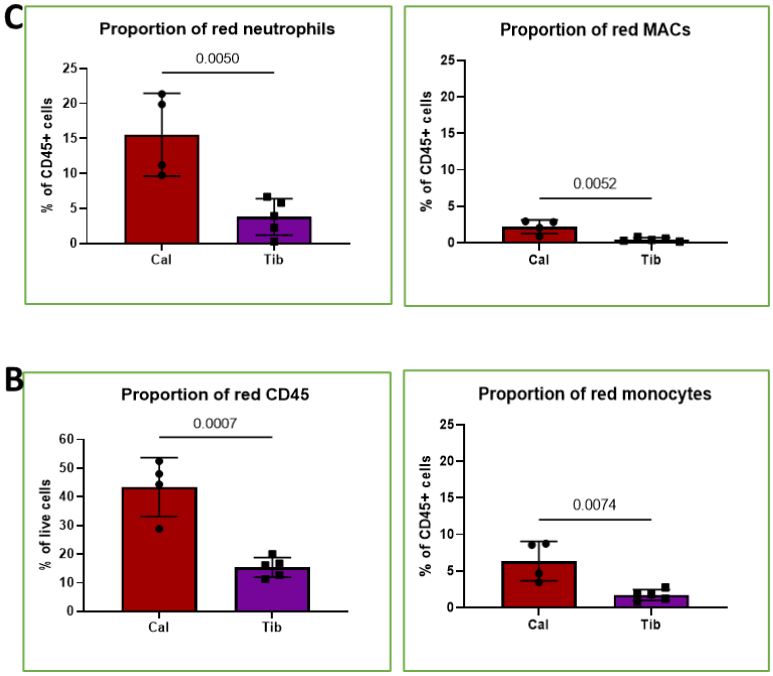


Figure 7: Baseline photoconversion of calvaria and tibias of KIKGR mice before retinal injury. A: Representative plots showing flow cytometric gating strategy for evaluation of photoconverted neutrophils, monocytes and MACs. B,C: Bar graphs showing differences in baseline photoconversion rates of (B) total CD45+ cells, monocytes, (C) neutrophils and MACs.

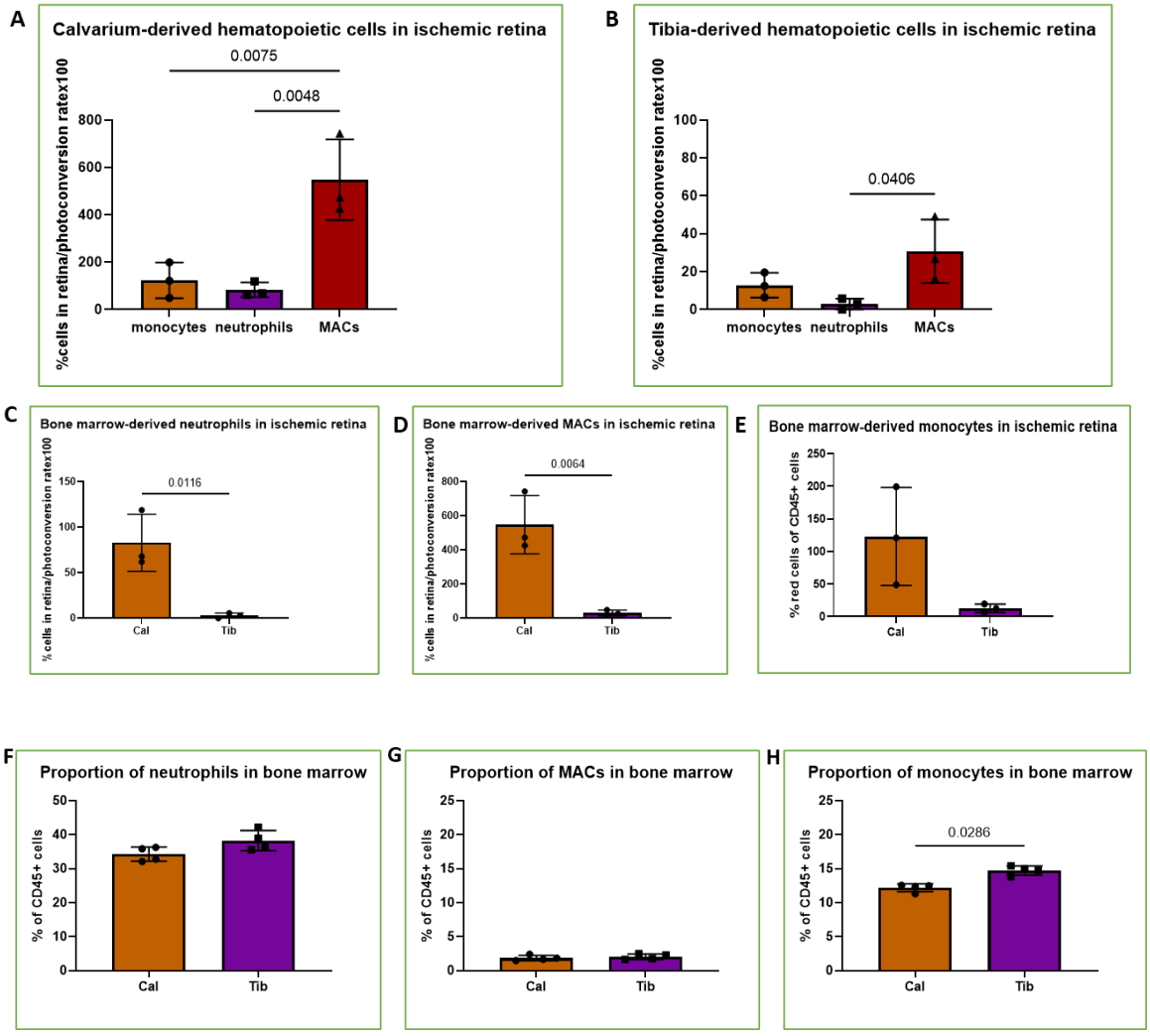


Figure 8: Relative contributions of calvarial and tibial marrows to neutrophils, MACs and monocytes in retina after ischemic injury. A-E: Bar graphs showing the normalized relative contribution of BM cells from the calvarium (A) and tibia (B) into the injured retina. The most robust response was observed from MACs in both calvarium and tibia photoconverted mice. However, (C-D) the calvarial marrow contributes significantly more neutrophils (C) and MACs (D) to the injured retina but not monocytes (E). F-H: Bar graphs showing the proportion of neutrophils, MACs and monocytes in calvarium (cal) and tibias (tib) of uninjured wild type mice.

HEMATOPOIETIC CELLS INFLUENCE VASCULAR DEVELOPMENT IN THE RETINA

by

BRIGHT ASARE-BEDIAKO, YVONNE ADU-AGYEIWAAH, ANTONIO ABAD, SERGIO LI CALZI, JASON L. FLOYD, RAM PRASAD, MARIANA DUPONT, RICHMOND ASARE-BEDIAKO, XOSE R. BUSTELO AND MARIA B. GRANT

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Format adapted and errata corrected for dissertation

ABSTRACT

Hematopoietic cells play a crucial role in the adult retina in health and disease. Monocytes, macrophages, microglia and myeloid angiogenic cells (MACs) have all been implicated in retinal pathology. However, the role that hematopoietic cells play in retinal development is understudied. The temporal changes in recruitment of hematopoietic cells into the developing retina and the phenotype of the recruited cells are not well understood. In this study, we used the hematopoietic cell-specific protein Vav1 to track and investigate hematopoietic cells in the developing retina. By flow cytometry and immunohistochemistry, we show that hematopoietic cells are present in the retina as early as P0, and include microglia, monocytes and MACs. Even before the formation of retinal blood vessels, hematopoietic cells localize to the inner retina where they eventually form networks that intimately associate with the developing vasculature. Loss of Vav1 lead to a reduction in the density of medium-sized vessels and an increased inflammatory response in retinal astrocytes. When pups were subjected to oxygen-induced retinopathy, hematopoietic cells maintained a close association with the vasculature and occasionally formed ‘frameworks’ for the generation of new vessels. Our study provides further evidence for the underappreciated role of hematopoietic cells in retinal vasculogenesis and the formation of a healthy retina.

Keywords: hematopoietic cells; developing retina; vasculogenesis; microglia; Vav1 knockout

INTRODUCTION

Hematopoietic cells are bone-marrow derived cells that become blood-borne myeloid and lymphoid cells and migrate to various tissues, participating both in immune responses and the maintenance of specific tissues [1–3]. Produced in the bone marrow in the adult homeostatic state [4], hematopoietic cells consist of undifferentiated, multipotent stem cells and progenitors responsible for their self-renewal and downstream mature immune cells [5]. Hematopoietic cells are involved in the maintenance of immune homeostasis in health [6] but are also activated in disease or stress to fight infection and aid in tissue repair. For example, in the steady state, monocytes patrol the vascular wall to assist in removal of infectious agents and other pathogens [7,8]. During injury or disease however, monocytes are recruited into the tissue parenchyma where they differentiate into macrophages with proinflammatory and anti-inflammatory activities [9–11]. In the adult retina, hematopoietic cells are immediately recruited following acute injury and home to sites of injury to facilitate tissue repair [2]. In the diabetic retina, hematopoietic cells accumulate in the lumen of the vasculature in response to increased vascular inflammation leading to capillary non-perfusion, endothelial cell loss and the eventual formation of acellular capillaries [12–14] and also extravasate from vessels to promote additional retinal pathology.

In murine development, hematopoietic cells first appear in the embryonic yolk sac between embryonic days 7 (E7) and 8 (E8) [15,16]. They are later detected in the aorta–gonads–mesonephros (E10.5), fetal liver (E11.5) and then the bone marrow (E14–16.5), which becomes the dominant hematopoietic site in the adult state. Concurrently, retina development begins around E10 and matures postnatally [17,18]. Multiple studies have

shown that some resident immune cells in the retina, typically microglia, are seeded in the eye from hematopoietic cells in the embryonic yolk sac, which migrate and proliferate in the retina [19,20]. However, a detailed characterization of the different phenotypes of hematopoietic cells in the developing retina is lacking, and the role that these cells play in retina development is understudied. The goal of this study was to investigate the timeline of the migration of different hematopoietic cells into the developing mouse retina postnatally and their contribution to the formation of a healthy adult retina.

MATERIALS AND METHODS

Animals

All animal experiments adhered to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham (APN-21223), the Bioethics Committee of the University of Salamanca (animal license #568) and the animal experimentation authorities of the autonomous Government of Castilla y León (Spain). They were treated humanely in accordance with standards described in the Guide for the Care and Use of Laboratory Animals, considering relevant national and European guidelines.

To generate Vav1-GFP mice, ROSAmTmG mice (Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J; The Jackson Laboratory) were crossed with Vav1-icre (B6.Cg-Commd10Tg(Vav1-icre)A2Kio/J; The Jackson Laboratory) mice. This allowed the expression of GFP under the Vav1 promoter, giving rise to GFP+ (Vav1) hematopoietic cells in subsequent progeny. Vav1-GFP newborns were euthanized at P0, P7, P14 and P21 and their retinas used for downstream experiments and analysis. Vav1–/– (Vav1 KO) mice were generated as described previously [21,22].

Flow Cytometry

Mice were perfused transcardially under isoflurane anesthesia with phosphate-buffered saline (PBS) to flush vessels before euthanasia. After euthanasia, eyes were enucleated into ice-cold phosphate-buffered saline (PBS). The corneas, lenses and hyaloid vasculatures were removed and the retinas isolated immediately. To dissociate

retinas in-to single cells, each retina was incubated in 1 ml of papain dissociation solution (Roche Diagnostics GmbH, REF#10108014001) prepared according to manufacturer's protocol for 30 min in a 37 °C water bath. Single cell suspensions were washed with FACs buffer on ice and then incubated with primary antibody cocktails for 45 min at 4°C in the dark. Antibodies used included CD45 Apc-eFluor780 (Invitrogen, Cat#47-0451-82), Ly6C Percp/Cy5.5 (Biolegend, Cat#128012), Ly6G (BV605 Biolegend, Cat#127639), CD11b PE-CF594 (BD Biosciences, Cat#562287), CD31 PE (Invitrogen, Cat#2114546) and Fixable viability Dye eFluor506 (Invitrogen, Cat# 65-0866-14). The cells were then washed with FACs buffer, resuspended and analyzed using BD FACSCelesta flow cytometer (BD Bio-sciences) and FlowJo™ v10.8 Software (BD Life Sciences). Gating of the different cell populations was performed as previously published [23].

Immunofluorescence of Flat-Mounted Retinas and Retinal Cross-Sections

Immunofluorescence of retinal cross-sections was performed as previously described [24]. Briefly, enucleated eyes were fixed in 4% paraformaldehyde solution for 15–30 min on ice. After removing the corneas and lenses, the posterior cups were incubated in 15% sucrose solution in phosphate-buffered saline (PBS) overnight at 4 °C, then transferred to 30% sucrose in PBS for 3–4 h. The samples were then embedded in optimal cutting temperature (O.C.T.) medium and immediately frozen on dry ice and stored at −80 °C until further processing. The sections were thawed at 37 °C for 15–30 min., washed in PBS and then permeabilized with 0.25% Triton-X in PBS for 5 min at room temperature. Sections were blocked with 10% normal horse serum in 1% bovine serum albumin (BSA) for 1 hr and incubated with primary antibody diluted in blocking

solution (1:100 dilution) over-night at 4 °C. Samples were then washed and incubated with the appropriate fluorescent-labeled secondary antibodies for 1 hr at room temperature, followed by washing with PBS. Sections were incubated with 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution (Invitrogen, Cat#D3571) for 5 min at room temperature. For retinal flat mounts, enucleated eyes were fixed in 4% paraformaldehyde solution for 90 min on ice. After fixation, the corneas, lenses and hyaloid vasculatures were removed and the whole retinas isolated, washed in PBS and incubated in blocking buffer for 3 hrs at room temperature. The samples were then incubated in primary antibodies overnight at 4°C, washed and incubated with secondary antibodies at room temperature for 4 h. The primary antibodies used were chicken anti-GFAP (Novus Biologicals, Cat# NBP1-05198) and rabbit anti-collagen IV (Abcam, Cat#19808). Finally, samples were washed and mounted with anti-fade mounting medium (Vector Laboratories, Cat# H-1000) for imaging. Images were obtained with a 40 X objective lens for cross-sections and 20 X for flat mounts and all experiments included negative controls in which the primary antibodies were replaced with blanks (blocking buffer). All analyzed images were acquired from the mid-periphery of the retina where we observed high density of vessels without interference from very large vessels at all time points.

Oxygen-Induced Retinopathy (OIR)

To study the impact of hematopoietic cells on abnormal retinal vascular development, we utilized the oxygen-induced retinopathy (OIR) model [25,26] as previously published [27]. Briefly, Vav1-GFP mice (P7) were placed with their nursing dams in a 75% oxygen atmosphere for 5 days. Mice were returned to normoxic

conditions at P12 and euthanized at P15 and P17, the timepoint for peak neovascularization or angiogenesis [28,29]. Retinas of the OIR mice were used in flat mount preparations as detailed above.

Quantification of Retinal Vascular Density Using VESGEN

VESGEN is a JAVA-based vascular analysis software program available from NASA (https://software.nasa.gov/software/ARC-17621-1) and operates as an ImageJ plugin [30,31]. In addition to the assessment of overall vascular densities, VESGEN also allows grouping and quantification of different generations of blood vessels in retinal images. For this study, 20 X images of retinal flat mounts were traced and binarized in Adobe Photoshop CC 2018 v19.1.2 (Adobe Systems Incorporated). The resulting binary images were loaded into VESGEN2D v1.11 for analysis. For this murine study, large-sized (macrovascular) vessels were defined as generations 1–3, medium-sized vessels as generations 4–6 and small-sized (microvascular) vessels were defined as generations 7 and greater [32].

RESULTS

Phenotype and Localization of Hematopoietic Cells in Healthy Developing Retina

Using mice that express GFP under the control of the promoter for Vav1, a gene that encodes a hematopoietic cell-specific signaling protein [33–36], we investigated the phenotype of hematopoietic cells in the retina during post-natal development by flow cytometry and immunohistochemistry. As shown in Figure 1, GFP+ hematopoietic cells were detected in the retina less than 24hrs after birth (P0). The proportion of these cells in the retina increased significantly after P7 but remained steady up to P21. We observed that the majority of GFP+ hematopoietic cells early in postnatal development were microglia (53.05%, 51.34%, 58.37%, for P0, P7, P14, respectively,). The proportion of hematopoietic cell-derived microglia was reduced at P21 (34.61%) but did not reach statistical significance ($p = 0.161$). In addition, we detected GFP+ myeloid angiogenic cells (MACs) and monocytes in the developing retina. While the levels of the angiogenesis-supporting MACs were steady from P0 to P21, we observed that the levels of monocytes fluctuated between early (P0 to P7), mid (P7 to P14) and late (P14 and P21) development.

The post-natal development of the retina is a tightly controlled process, such that the different neuronal cell types are being specialized into mature neurons while vasculogenesis occurs concurrently. We investigated which retinal layers showed hematopoietic cell recruitment during development. As shown in Figure 2, we observed that GFP+ hematopoietic cells were recruited into the inner retina (from inner limiting membrane to outer plexiform layer) at all the time points investigated, while the outer

retina was largely devoid of hematopoietic cells, except for dendritic processes of a few cells.

Hematopoietic Cells and Retinal Angiogenesis and Inflammation

One of the main events that occurs in the murine retina postnatally is the final formation of the vascular network. Using flat mounted retina and immunohistochemistry, we observed that GFP+ hematopoietic cells form networks that closely associated with the developing vasculature (Figure 3) and occasionally assimilated into vessels (Figure 4). At P7, we observed that GFP+ hematopoietic cells occasionally formed retinal vessels (Figure 4A, inset). This suggests that hematopoietic cells may play a role in retinal vasculogenesis during development.

We next examined the retinas of Vav1 knockout (KO) mice to evaluate the impact of loss of functional hematopoietic cells on retinal vascular development and used VESGEN for analysis of the vasculature. As shown in Figure 5, we observed that Vav1 KO mice have a significantly reduced vessel number density at 1 month of age compared to wild-type (WT) mice (0.1592/pixel2 ± 0.011 vs. 0.2337/pixel2 ± 00025, p = 0.0018). We observed a significant difference in vessel density in the medium-sized vessels but not the large vessels or microvessels. We also examined the expression of glial fibrillary acidic protein (GFAP) in the developing retina. GFAP is normally expressed by retinal astrocytes but is increased with inflammation. We observed that GFAP expression was significantly elevated in Vav1 KO mice compared to WT (29.48a.u ± 10.09 vs. 14.34 ± 5.91, p = 0.0412), supporting an increased inflammatory response in retinal astrocytes of Vav1 KO mice. Finally, we examined 7-month-old Vav1 KO mice and found that they had reduced vascular density (Figure S2D) but not significant difference in GFAP

expression (Figure S2E) compared to controls, suggesting that while the KO retinas recover from the increase in inflammation observed early after birth, the loss of vascular density persists.

Hematopoietic Cells in Abnormal Retinal Vascular Development

We used the OIR model to investigate the role of hematopoietic cells in retinal vascular repair during abnormal retinal development. In the OIR, exposure to high oxygen followed by a return to normoxia creates a relative hypoxic environment in the retina that leads to abnormal vascular development. Similar to WT retinas, we observed that GFP+ hematopoietic cells were recruited into the retina and were closely associated with the vasculature (Figure 6). In the retinas of mice subjected to OIR, hematopoietic cells aggregated and elongated to form vascular branches (Figure 6D) and formed new vessels or repaired damaged vessels at P17.

DISCUSSION

The major findings of this work include that hematopoietic cells are recruited early in development and participate in the formation of the healthy retina mainly as microglia and by facilitating retinal angiogenesis. GFP+ cells in the retina appear as early as less than 24 h after birth (P0) and reach steady levels from P7 to P21 with the majority of these cells becoming microglia. We show that hematopoietic cells consistently align with the developing vasculature, potentially providing paracrine support to aid vasculogenesis in the healthy developing retina and also for vascular repair in abnormal vascular development.

The involvement of hematopoietic cells in maintenance of retinal health is widely appreciated in the adult retina. Hematopoietic cells are mobilized from the bone marrow and recruited into injured or diseased retina to orchestrate tissue repair, inflammation and/or cell death [37–39]. However, the role that these cells play in the development of the retina and the temporal dynamics of their recruitment during postnatal development is understudied. We used transgenic mice that express GFP in the hematopoietic cell-specific protein Vav1 [35] to study the temporal changes in the recruitment of hematopoietic cells into the developing retina postnatally. GFP+ hematopoietic cells appeared in the retina as early as P0 and reached steady levels from P7 to P21 (Figure 1). Retinal microglia were the primary cell type derived from hematopoietic cells. Microglia arise from primitive hematopoietic cells in the embryonic yolk sac (which become the resident microglia) and previous studies have identified microglia in the retina prenatally [10,20]. We observed myeloid leukocytes (ML) in the developing retina, and they were significantly reduced after P7. Of myeloid leukocytes in the retina, the levels of MACs

remained consistent up to P21 while the number of monocytes fluctuated. The initial relatively high levels of ML (predominantly monocytes) are likely in response to neonatal stress [40,41] after exposure of the newborn to a new oxygenated environment, which influences the mobilization of hematopoietic cells [42]. Given that there is no blood-retinal barrier at birth in mice, circulating myeloid leukocytes enter the retina unrestricted. However, monocytes and other myeloid leukocytes only remain in the retina for a few days and are replaced [23]. Monocytes patrol the retina to protect against infection [7] and facilitate the activation of tissue resident microglia and macrophages to phagocytose [7,19] dead or misplaced cells as the developing retina organizes into distinct layers. The fluctuating levels of these cells in the developing retina is indicative of the dynamics of recruitment and removal of myeloid cells protecting the retina by facilitating the elimination of dead cells or unwanted cells every few days.

Whereas vascular wall-derived endothelial colony forming cells (ECFCs) are the main cells capable of forming blood vessels de novo, MACs are hematopoietic cells that provide paracrine support to ECFCs during angiogenesis [43,44]. Given that the newborn mouse retina is devoid of blood vessels, the MACs are likely recruited to facilitate formation of blood vessels de novo and their organization into layers in the developing retina postnatally. We observed that GFP+ hematopoietic cells were distributed over the entire retina without preference for central or peripheral retina as shown in Figure S1. Other studies have shown bone marrow-derived cells enter the retina through the optic nerve head and ciliary body [45]. With no blood vessels and no blood-retinal barrier until later in development, the cells enter the retina mainly through the optic nerve head prior to P0 (Figure S2A), and also through the choroid (Figure 2E) and migrate towards the

inner retina and periphery as vasculogenesis begins and progresses. The recruited hematopoietic cells predominantly occupied the inner retina, from the inner limiting membrane to the outer plexiform layer (Figure 2). Santos et al. [20] observed similar localization of microglia in the developing retina, which is what we observed in our study. Interestingly, normal vascular development in the mouse retina is also confined to the inner retina [46,47]. We observed that GFP+ hematopoietic cells organized into connected networks that closely paralleled and occasionally incorporated into the developing vasculature (Figure 3,4). Retinal astrocytes form the framework that guides the structural organization of blood vessels in the developing retina [48,49]. However, it has been shown that hematopoietic cells are capable of targeting retinal astrocytes to promote both vasculogenesis and retinal vascular repair [50,51]. MACs secrete paracrine factors that recruit and guide ECFCs to areas of vasculogenesis [43]. Hematopoietic cells provide pro-angiogenic factors as well as structural support that promote angiogenesis [52]. Thus, our study adds to the increasing evidence of hematopoietic cells as key regulators of retinal vascular development.

To investigate further the potential impact of hematopoietic cells on retinal vascular development, we used VESGEN to analyze the retinal vasculature of Vav1 KO mice. Vav1 is a signal transducer that mediates cytoskeletal rearrangement required for activation and mobilization of hematopoietic cells. Phosphorylation of Vav1 is essential for cell signaling and activation of receptors in the hematopoietic system [33,53,54]. We observed a reduction in retinal vascular density in Vav1 KO mice compared to controls (Figure 5), predominantly in the medium-sized vessels (generations 4 to 6). Previous studies have shown that loss of Vav1 leads to a reduction in lymphocytes [21],

particularly T cells [22,55]. Interestingly, Deliyanti et al. observed that expansion of regulatory T cells alleviates pathological angiogenesis [56], supporting the role that hematopoietic cells play in the formation and maintenance of blood vessels. In addition, Vav1 KO mice showed increased inflammation as evidenced by an increase in GFAP expression in retinal astrocytes- key modulators of retinal angiogenesis in development [50]. Thus, lack of and/or a dysfunction of hematopoietic cells in the developing retina is associated with retinal astrocyte pathology which negatively impacts vascular development. Reduced retinal vascular density is a known indicator of poor retinal function and is associated with retinal pathologies [51,57,58]. Reduced vascular density in Vav1KO mice therefore implies a reduction in retinal blood supply of oxygen and nutrients and inadequate removal of metabolic wastes, which affects retinal function. This, in part, could account for the increase in inflammation (GFAP expression) observed in Vav1 KO retinas.

Retinopathy of prematurity (ROP) is characterized by abnormal retinal neurovascular growth in preterm newborns, caused by the exposure of the of immature retina to hyperoxic-to-hypoxic conditions leading to aberrant retinal vascularization [59–62]. The murine OIR model has been used to recapitulate and investigate the pathogenesis of ROP [63–65]. The OIR model, like most vascular injury models, leads to infiltration of hematopoietic cells into the retina [66,67], and intravitreally administered hematopoietic cells are able to target the retinal vasculature to reverse abnormal retinal vascular development [27]. We subjected Vav1-GFP mice to OIR injury to track the response of endogenous hematopoietic cells in pathological vascular development. Hematopoietic cells maintained close association with blood vessels as observed in non-

OIR retinas (Figure 6A–C). Hematopoietic cells incorporated into blood vessels and occasionally formed ‘channels’ (Figure 6D) as framework for the formation of new vessels. These findings support that hematopoietic cells may play a vital role in orchestrating retinal vasculogenesis during development.

Our study has limitations. Even though Vav1 is widely known to be expressed in hematopoietic cells, it can infrequently be turned on in endothelial cells [68]. Nonetheless, given the overwhelming evidence in the literature that Vav1 cells are hematopoietic [55,69,70] and the retinal phenotype of the Vav1KO mice (Figure S2B), our data supports the role that hematopoietic cells play in retinal development. Data from 7-month-old Vav1 KO mice and controls (Figure S2D,E) shows that at 7 months of age, Vav1 KO retinas have a significantly reduced vascular density (Figure S2D) but no significant difference in GFAP expression (Figure S2E). Thus, while the Vav1 KO retinas recover from the increase in inflammation experienced early after birth, the loss in vascular density persists beyond the resolution of inflammation. This suggests that the reduction in vascular density is more likely due to the loss of hematopoietic cells in the retina and not secondary to inflammation. In summary, our data supports that hematopoietic cells play a vital role in orchestrating retinal vasculogenesis during development. Further studies should also seek to correlate the reduced vascular density observed in these mice with results from physiological tests of functions such as electroretinography.

CONCLUSIONS

Our study characterizes hematopoietic cells recruited into the developing retina and the possible roles of these cells in the formation of a healthy retina. In addition to microglia which are seeded prenatally, monocytes and MACs participate in the growth and development of the retina. Hematopoietic cells maintain a close association with the developing vasculature, contributing to the regulation of angiogenesis either directly or indirectly by targeting retinal astrocytes. Further investigation is required to elucidate the interplay between hematopoietic cells, astrocytes and endothelial colony forming cells in vasculogenesis.

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cells11203207/s1, Figure S1: Distribution of hematopoietic cells in retina development, Figure S2: Hematopoietic cells play a role in vasculogenesis in retina development.

AUTHOR CONTRIBUTIONS

Conceptualization, M.B.G., X.R.B. and B.A.-B.; methodology, M.B.G., X.R.B. and B.A.-B.; software, B.A.-B. and M.D., R.A.-B.; validation, M.B.G., X.R.B., S.L.C., A.A. and B.A.-B.; formal analysis, M.B.G., B.A.-B., S.L.C. and R.P.; investigation, B.A.-B., Y.A.-A., A.A., S.L.C., R.P., J.L.F., M.D., and R.A.-B.; resources, M.B.G. and X.R.B.; data curation, B.A.-B., S.L.C., Y.A.-A., R.A.-B., J.L.F.; writing—original draft preparation, M.B.G., X.R.B. and B.A.-B.; writing—review and editing, M.B.G., X.R.B., B.A.-B., Y.A.-A., A.A., S.L.C., R.P., J.L.F., M.D., and R.A.-B.; visualization, B.A.-B., S.L.C.,

R.P., R.A.-B., ; supervision, M.B.G. and X.R.B.; project administration, M.B.G., X.R.B. and S.L.C..; funding acquisition, M.B.G. and X.R.B. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

1. Alomari, M. et al. Role of lipid rafts in hematopoietic stem cells homing, mobilization, hibernation, and differentiation. Cells 2019, 8, 630.

2. Grant, M.B.; et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat. Med. 2002, 8, 607–612.

3. Udupa, K. Age and the hematopoietic system. J. Am. Geriatr. Soc. 1986, 34, 448–454.

4. Scala, S.; Aiuti, A. In vivo dynamics of human hematopoietic stem cells: Novel concepts and future directions. Blood Adv. 2019, 3, 1916–1924.

5. Monga, I.; Kaur, K.; Dhanda, S.K. Revisiting hematopoiesis: Applications of the bulk and single-cell transcriptomics dissecting transcriptional heterogeneity in hematopoietic stem cells. Brief. Funct. Genom. 2022, 21, 159–176.

6. Abkowitz, J.L.; et al. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. Blood 2003, 102, 1249–1253.

7. Auffray, C.; et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007, 317, 666–670.

8. Carlin, L.M.; et al. Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. Cell 2013, 153, 362–375.

9. Arnold, L.; et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J. Exp. Med. 2007, 204, 1057–1069.

10. Ginhoux, F.; Jung, S. Monocytes and macrophages: Developmental pathways and tissue homeostasis. Nat. Rev. Immunol. 2014, 14, 392–404.

11. Shechter, R.; et al. Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. PLoS Med. 2009, 6, e1000113.

12. Miyamoto, K.; et al. Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via in-tercellular adhesion molecule-1 inhibition. Proc. Natl. Acad. Sci. 1999, 96, 10836–10841.

13. Barouch, F.C.; et al. Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes. Investig. Ophthalmol. Vis. Sci. 2000, 41, 1153–1158.

14. Joussen, A.M.; et al. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. Am. J. Pathol. 2001, 158, 147–152.

15. Luis, T.; Killmann, N.; Staal, F. Signal transduction pathways regulating hematopoietic stem cell biology: Introduction to a series of Spotlight Reviews. Leukemia 2012, 26, 86–90.

16. Orkin, S.H.; Zon, L.I. Hematopoiesis: An evolving paradigm for stem cell biology. Cell, 2008, 132, 631–644.

17. Telegina, D.V.; et al. Features of retinal neurogenesis as a key factor of age-related neurodegeneration: Myth or reality? Int. J. Mol. Sci. 2021, 22, 7373.

18. Rapaport, D.H.; et al. Timing and topography of cell genesis in the rat retina. J. Comp. Neurol. 2004, 474, 304–324.

19. Silverman, S.M.; Wong, W.T. Microglia in the retina: Roles in development, maturity, and disease. Annu. Rev. Vis. Sci. 2018, 4, 45–77.

20. Santos, A.M.; et al. Embryonic and postnatal development of microglial cells in the mouse retina. J. Comp. Neurol. 2008, 506, 224–239.

21. Tarakhovsky, A.; et al. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature 1995, 374, 467–470.

22. Turner, M.; et al. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity 1997, 7, 451–460.

23. Abcouwer, S.F.; et al. Inflammatory resolution and vascular barrier restoration after retinal ischemia reperfusion injury. J. Neuroinflammation 2021, 18, 186.

24. Asare-Bediako, B.; et al. Characterizing the retinal phenotype in the high-fat diet and western diet mouse models of predi-abetes. Cells 2020, 9, 464.

25. Madan, A.; Penn, J.S. Animal models of oxygen-induced retinopathy. Front. Biosci. A J. Virtual Libr. 2003, 8, d1030-43.

26. Smith, L.E.H.; et al. Oxygen-induced retinopathy in the mouse. Investig. Ophthalmol. Vis. Sci. 1994.

27. Calzi, S.L.; et al. Progenitor cell combination normalizes retinal vascular development in the oxygen-induced retinopathy (OIR) model. JCI Insight 2019, 4, e129224.

28. Zhou, L.; et al. Imatinib ameliorated retinal neovascularization by suppressing PDGFR-α and PDGFR-β. Cell. Physiol. Biochem. 2018, 48, 263–273.

29. Joe, S.G.; et al. Anti-angiogenic effect of metformin in mouse oxygen-induced retinopathy is mediated by reducing levels of the vascular endothelial growth factor receptor Flk-1. PLoS ONE 2015, 10, e0119708.

30. Vickerman, M.B.; et al. VESGEN 2D: Automated, user–interactive software for quantification and mapping of angiogenic and lymphangiogenic trees and networks. Anat. Rec. Adv. Integr. Anat. Evol. Biol. Adv. Integr. Anat. Evol. Biol. 2009, 292, 320–332.

31. DuPont, M.; et al. Retinal vessel changes in pulmonary arterial hypertension. Pulmonary Circulation, 2022, 12, e12035.

32. Gil, C.-H.; et al. Specific mesoderm subset derived from human pluripotent stem cells ameliorates microvascular pathology in type 2 diabetic mice. Sci. Adv. 2022, 8, eabm5559.

33. Katzav, S. Flesh and blood: The story of Vav1, a gene that signals in hematopoietic cells but can be transforming in human malignancies. Cancer Lett. 2007, 255, 241–254.

34. Katzav, S.; Martin-Zanca, D.; Barbacid, M. Vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. EMBO J. 1989, 8, 2283–2290.

35. Katzav, S. Vav1: A Dr. Jekyll and Mr. Hyde protein–good for the hematopoietic system, bad for cancer. Oncotarget 2015, 6, 28731.

36. Bustelo, X.; et al. Developmental expression of the vav protooncogene. Cell Growth Differ. Mol. Biol. J. Am. Assoc. Cancer Res. 1993, 4, 297–308.

37. Liu, Y.; et al. Reversible retinal vessel closure from VEGF-induced leukocyte plugging. JCI Insight 2017, 2, e95530.

38. Alt, C.; et al. In vivo tracking of hematopoietic cells in the retina of chimeric mice with a scanning laser ophthalmoscope. IntraVital 2012, 1, 132–140.

39. Xu, H.; et al. Turnover of resident retinal microglia in the normal adult mouse. Glia 2007, 55, 1189–1198.

40. Gitau, R.; et al. Umbilical cortisol levels as an indicator of the fetal stress response to assisted vaginal delivery. Eur. J. Obstet. Gynecol. Reprod. Biol. 2001, 98, 14–17.

41. Vogl, S.; et al. Mode of delivery is associated with maternal and fetal endocrine stress response. BJOG: Int. J. Obstet. Gynaecol. 2006, 113, 441–445.

42. Bui, K.C.T.; et al. Circulating hematopoietic and endothelial progenitor cells in newborn infants: Effects of gestational age, postnatal age and clinical stress in the first 3 weeks of life. Early Hum. Dev. 2013, 89, 411–418.

43. Banno, K.; Yoder, M.C. Tissue regeneration using endothelial colony-forming cells: Promising cells for vascular repair. Pediatr. Res. 2018, 83, 283–290.

44. Yoder, M.C.; et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell prin-cipals. Blood 2007, 109, 1801–1809.

45. Kaneko, H.; et al. Characteristics of bone marrow–derived microglia in the normal and injured retina. Investig. Ophthalmol. Vis. Sci. 2008, 49, 4162–4168.

46. Liu, C.-H.; et al. Animal models of ocular angiogenesis: From development to pathologies. FASEB J. 2017, 31, 4665.

47. Dorrell, M.I.; Friedlander, M. Mechanisms of endothelial cell guidance and vascular patterning in the developing mouse retina. Prog. Retin. Eye Res. 2006, 25, 277–295.

48. Paisley, C.E.; Kay, J.N. Seeing stars: Development and function of retinal astrocytes. Dev. Biol. 2021, 478, 144–154.

49. Kubota, Y.; Suda, T. Feedback mechanism between blood vessels and astrocytes in retinal vascular development. Trends Cardiovasc. Med. 2009, 19, 38–43.

50. Otani, A.; et al. Bone marrow–derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. Nat. Med. 2002, 8, 1004–1010.

51. Friedlander, M.; et al. Progenitor cells and retinal angiogenesis. Angiogenesis 2007, 10, 89–101.

52. Calzi, S.L.; et al. EPCs and pathological angiogenesis: When good cells go bad. Microvasc. Res. 2010, 79, 207–216.

53. Caloca, M.J.; et al. Vav mediates Ras stimulation by direct activation of the GDP/GTP exchange factor Ras GRP1. EMBO J. 2003, 22, 3326–3336.

54. Rodríguez-Fdez, S.; Bustelo, X.R. The Vav GEF family: An evolutionary and functional perspective. Cells 2019, 8, 465.

55. Tybulewicz, V.L.; et al. Vav1: A key signal transducer downstream of the TCR. Immunol. Rev. 2003, 192, 42–52.

56. Deliyanti, D.; et al. Foxp3+ Tregs are recruited to the retina to repair pathological angiogenesis. Nat. Commun. 2017, 8, 748.

57. Czakó, C.; et al. Decreased retinal capillary density is associated with a higher risk of diabetic retinopathy in patients with diabetes. Retina 2019, 39, 1710–1719.

58. Wang, H.; et al. Quantitative analyses of retinal vascular area and density after different methods to reduce VEGF in a rat model of retinopathy of prematurity. Investig. Ophthalmol. Vis. Sci. 2014, 55, 737–744.

59. Hellström, A.; Smith, L.E.; Dammann, O. Retinopathy of prematurity. Lancet 2013, 382, 1445–1457.

60. Chen, J.; Smith, L.E. Retinopathy of prematurity. Angiogenesis 2007, 10, 133–140.

61. Hartnett, M.E.; Penn, J.S. Mechanisms and management of retinopathy of prematurity. New Engl. J. Med. 2012, 367, 2515–2526.

62. Early Treatment for Retinopathy of Prematurity Cooperative Group. The incidence and course of retinopathy of prematurity: Findings from the early treatment for retinopathy of prematurity study. Pediatrics 2005, 116, 15–23.

63. Connor, K.M.; et al. Quantification of oxygen-induced retinopathy in the mouse: A model of vessel loss, vessel regrowth and pathological angiogenesis. Nat. Protoc. 2009, 4, 1565–1573.

64. Pan, H.; et al. Molecular targeting of antiangiogenic factor 16K hPRL inhibits oxygen-induced retinopathy in mice. Investig. Ophthalmol. Vis. Sci. 2004, 45, 2413–2419.

65. Neu, J.; et al. The dipeptide Arg-Gln inhibits retinal neovascularization in the mouse model of oxygen-induced retinopathy. Investig. Ophthalmol. Vis. Sci. 2006, 47, 3151–3155.

66. Gao, X.; et al. Macrophages promote vasculogenesis of retinal neovascularization in an oxygen-induced retinopathy model in mice. Cell Tissue Res. 2016, 364, 599–610.

67. Li Calzi, S.; et al. Endothelial progenitor dysfunction in the pathogenesis of diabetic retinopathy: Treatment concept to correct diabetes-associated deficits. EPMA J. 2010, 1, 88–100.

68. Hong, J.; et al. Vav1 is Essential for HIF-1α Activation via a Lysosomal VEGFR1-Mediated Degradation Mechanism in En-dothelial Cells. Cancers 2020, 12, 1374.

69. Bustelo, X.R. Regulatory and signaling properties of the Vav family. Mol. Cell. Biol. 2000, 20, 1461–1477.

70. Turner, M.; Billadeau, D.D. VAV proteins as signal integrators for multi-subunit immune-recognition receptors. Nat. Rev. Immunol. 2002, 2, 476–486.

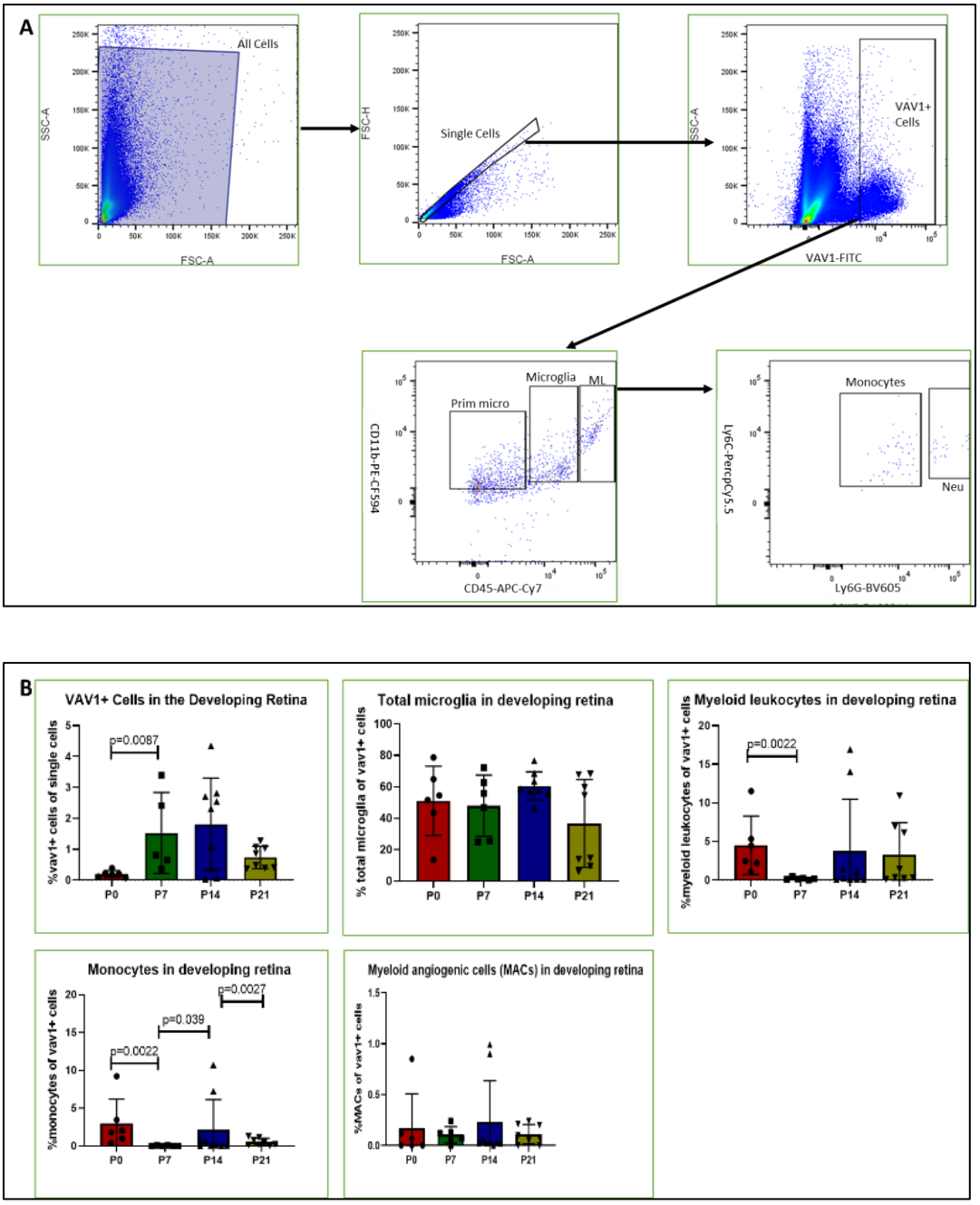
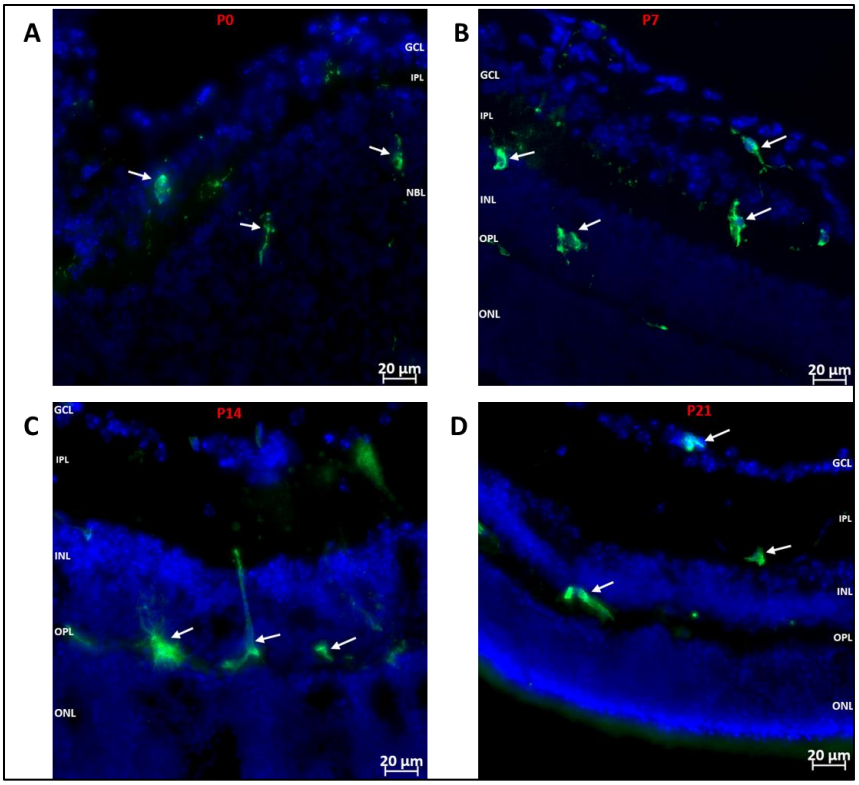


Figure 1. Hematopoietic cells in developing retina (A). Representative plots showing flow cytometric analysis gates used to quantify cells within the retina. After gating for single cells, events were sub-gated into GFP+(Vav1) cells, GFP+/CD11b+/CD45high myeloid leukocytes (ML), GFP+/CD11b+/CD45low microglia and GFP+/CD11b+/CD45- 'primitive' microglia. ML were sub-gated into Ly6C+Ly6G- monocytes, and CD31+ myeloid angiogenic cells (MACs). Panel (B) shows quantification for each of these populations at P0, P7, P14 and P21 as indicated.



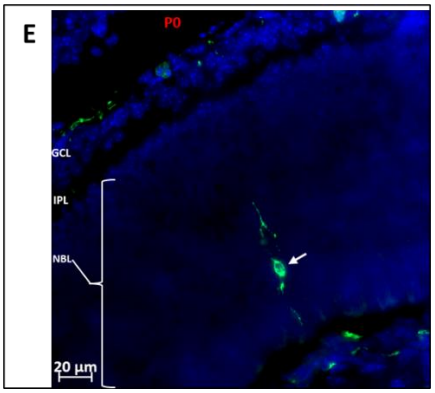


Figure 2. Localization of hematopoietic cells in developing retina. (A–D): Representative images showing localization of GFP+ (green) hematopoietic cells in the retina at P0, P7, P14 and P21. The cells predominantly migrate to the inner retina (inner limiting membrane to outer plexiform layer). (E): Representative image showing the occasional localization of a hematopoietic cell in the outer retina. A GFP+ cell is observed in the developing outer retina extending dendritic processes towards the RPE layer and inner retina. GFP+ cells are also observed arriving at the posterior retina via the choroid. Nuclei were stained with DAPI (blue). GCL: Ganglion cell layer; IPL: inner plexiform layer; NBL: neuroblast layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

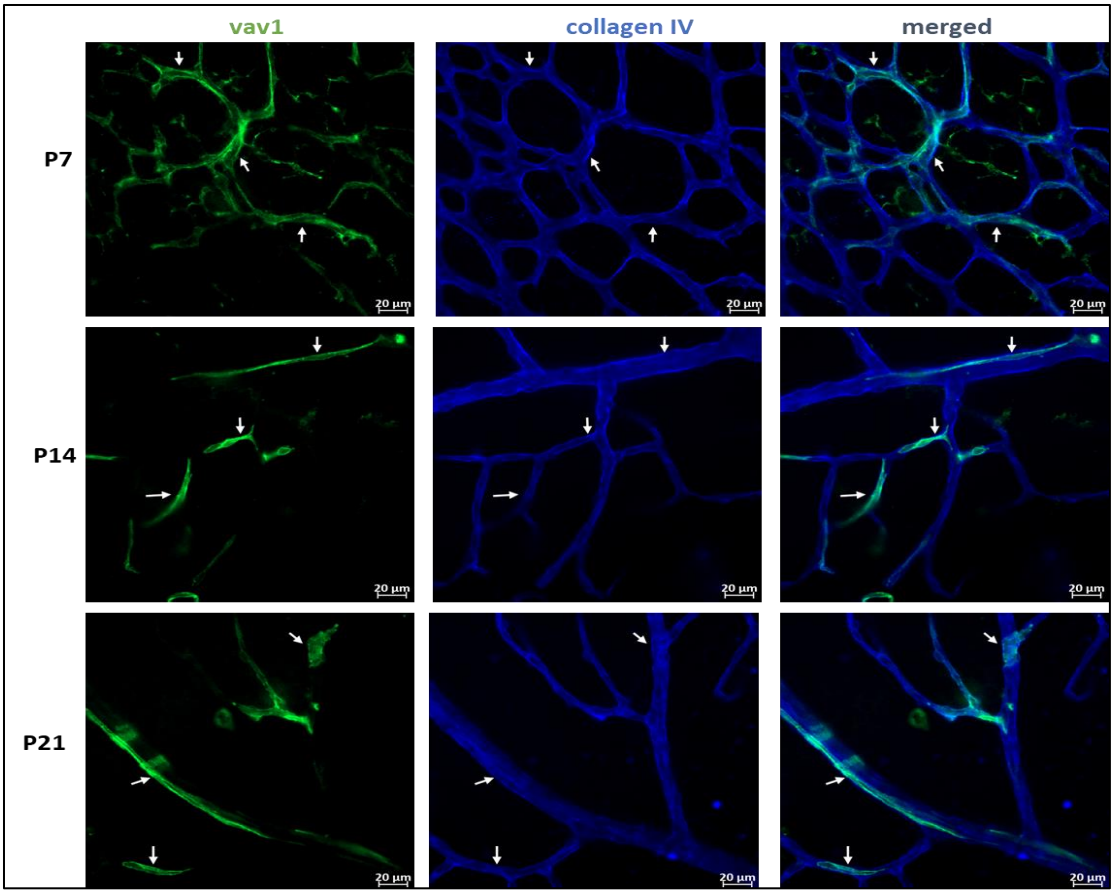
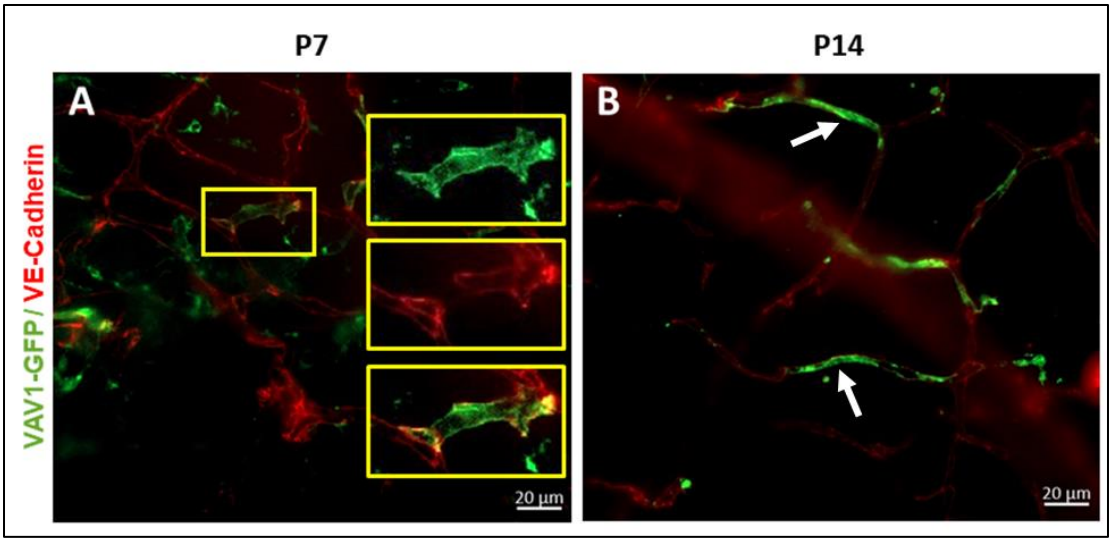


Figure 3. Hematopoietic cells and vascular development in the retina. Representative images showing the close association between GFP+ hematopoietic cells and the developing vasculature in the mouse retina. In early retinal vascular development (P7), hematopoietic cells in the retina form networks (P7, arrows) that parallel the developing vasculature, providing support to the vessels. Remnants of these networks persist throughout development (P14-P21, arrows).



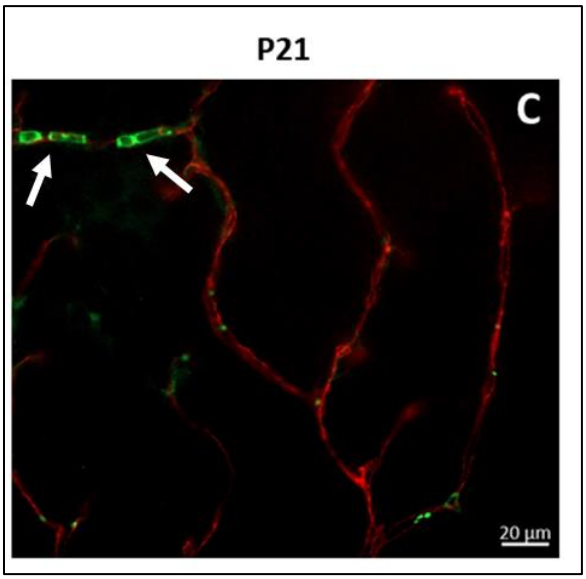
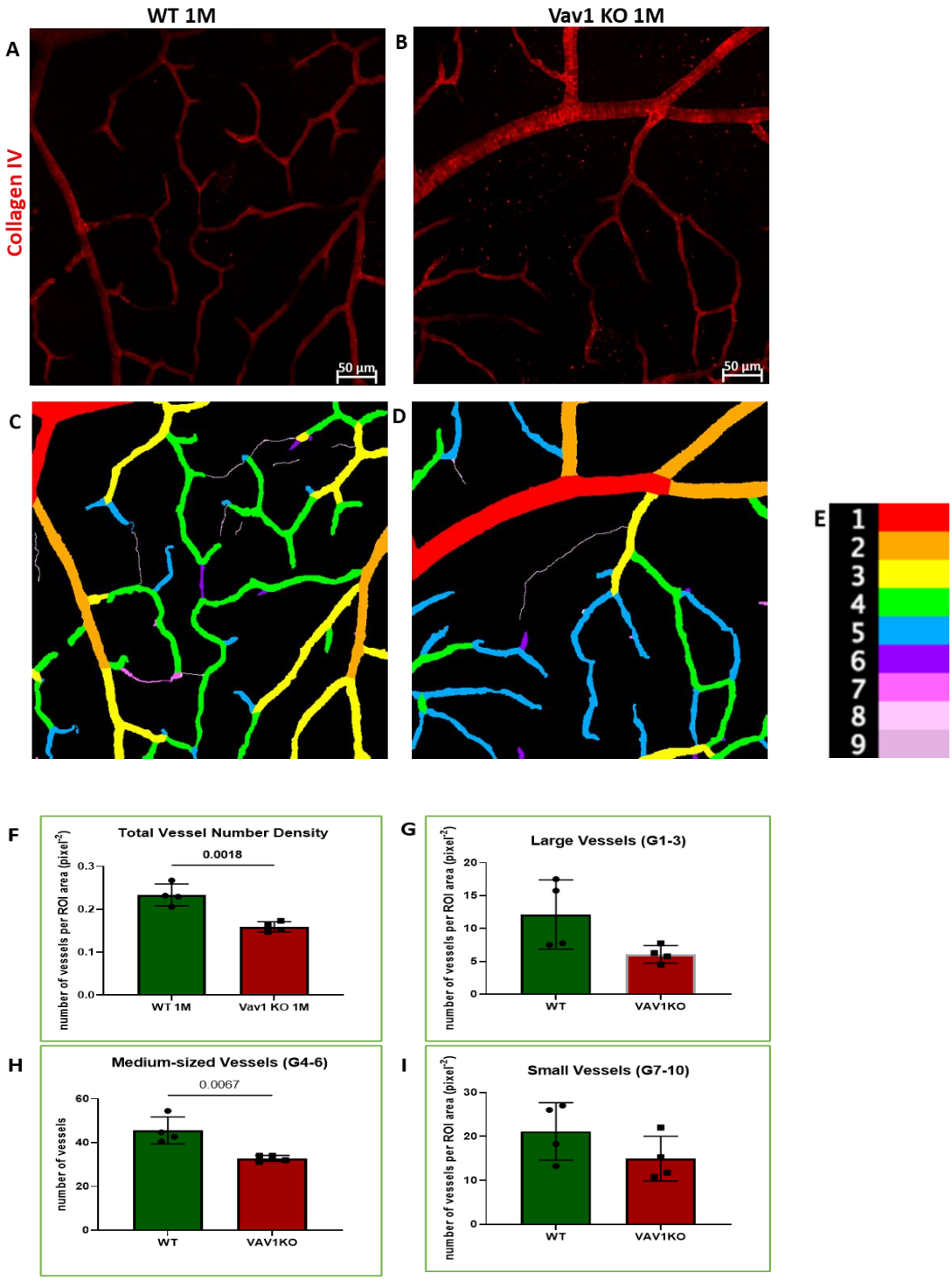
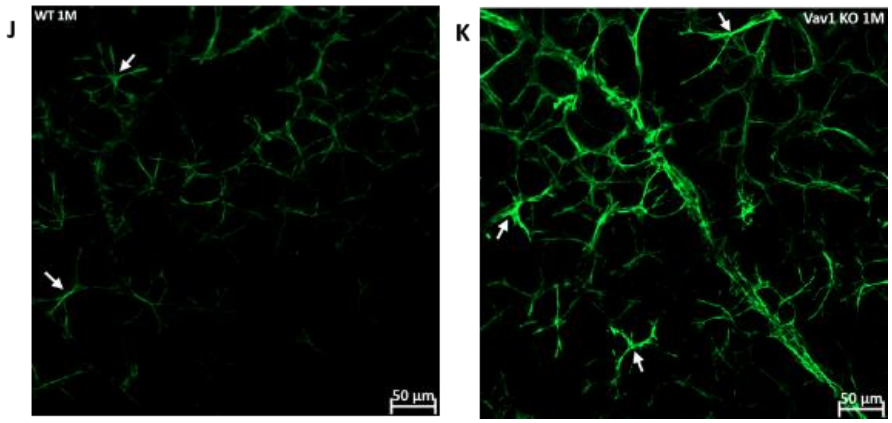


Figure 4. Hematopoietic cells in retinal vascular development. Hematopoietic cells support an-giogenesis in the developing retina. GFP+ hematopoietic cells provide a framework for the completion of a branch vessel in the retina at P7 ((A), inset) and are occasionally incorporated into the developing vasculature at P14 ((B), white arrow) and P21 ((C), white arrow) for the formation of healthy/normal vessels.


WT 1M
Vav1 KO 1M
A
B
Collagen IV
50 µm
50 µm
C
D
E
1
2
3
4
5
6
7
8
9
F
Total Vessel Number Density
0.0018
number of vessels per ROI area (pixel-2)
0.3
0.2
0.1
0.0
WT 1M
Vav1 KO 1M
G
Large Vessels (G1-3)
number of vessels per ROI area (pixel-2)
20
15
10
5
0
WT
VAV1KO
H
Medium-sized Vessels (G4-6)
0.0067
number of vessels
60
40
20
0
WT
VAV1KO
I
Small Vessels (G7-10)
number of vessels per ROI area (pixel-2)
30
20
10
0
WT
VAV1KO



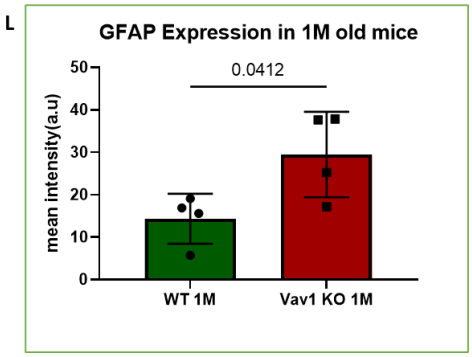


Figure 5. Loss of hematopoietic cells in the developing retina delays retinal vascular development and increases inflammation. Representative images (A,B) showing retinal flat mounts of WT and Vav1 KO retinas stained with collagen IV which labels the vasculature, and the corresponding outputs from VESGEN (C,D) showing the different vessel generations color coded (E) from 1 − 9. (F − I) Graphs showing VESGEN quantifications comparing total vessel number density, large vessel, medium-sized vessel and small vessel densities between the two groups. (J,K) Representative images showing GFAP expression (green) in the retinas of WT and Vav1 KO mice and the cor-responding quantification in (L). White arrows indicate retinal astrocytes.

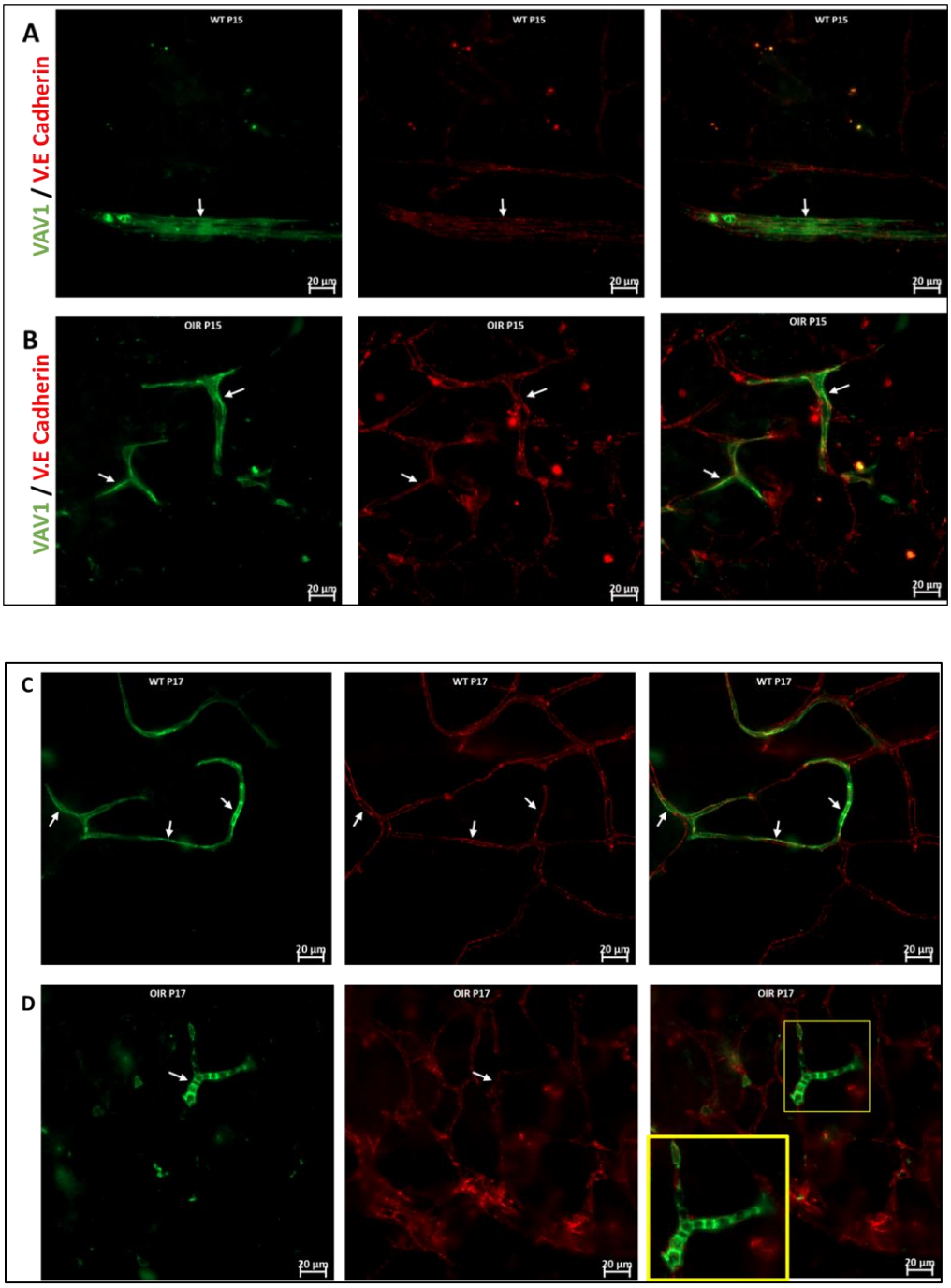


Figure 6. Hematopoietic cells in abnormal vascular development and repair. Representative images (A,B) showing retinal flat mounts of Vav1-GFP without OIR (WT) and with OIR (OIR) at 3 days (P15; (A,B)) and 5 days (P17; (C,D)) post OIR. At P15, GFP+ hematopoietic cells are observed forming close association with the vasculature in both models (white arrows). At P17, the peak of neovascularization, recruited GFP+ hematopoietic cells are observed to align/aggregate ((D), inset) to remodel or repair a damaged vessel in the developing retina.

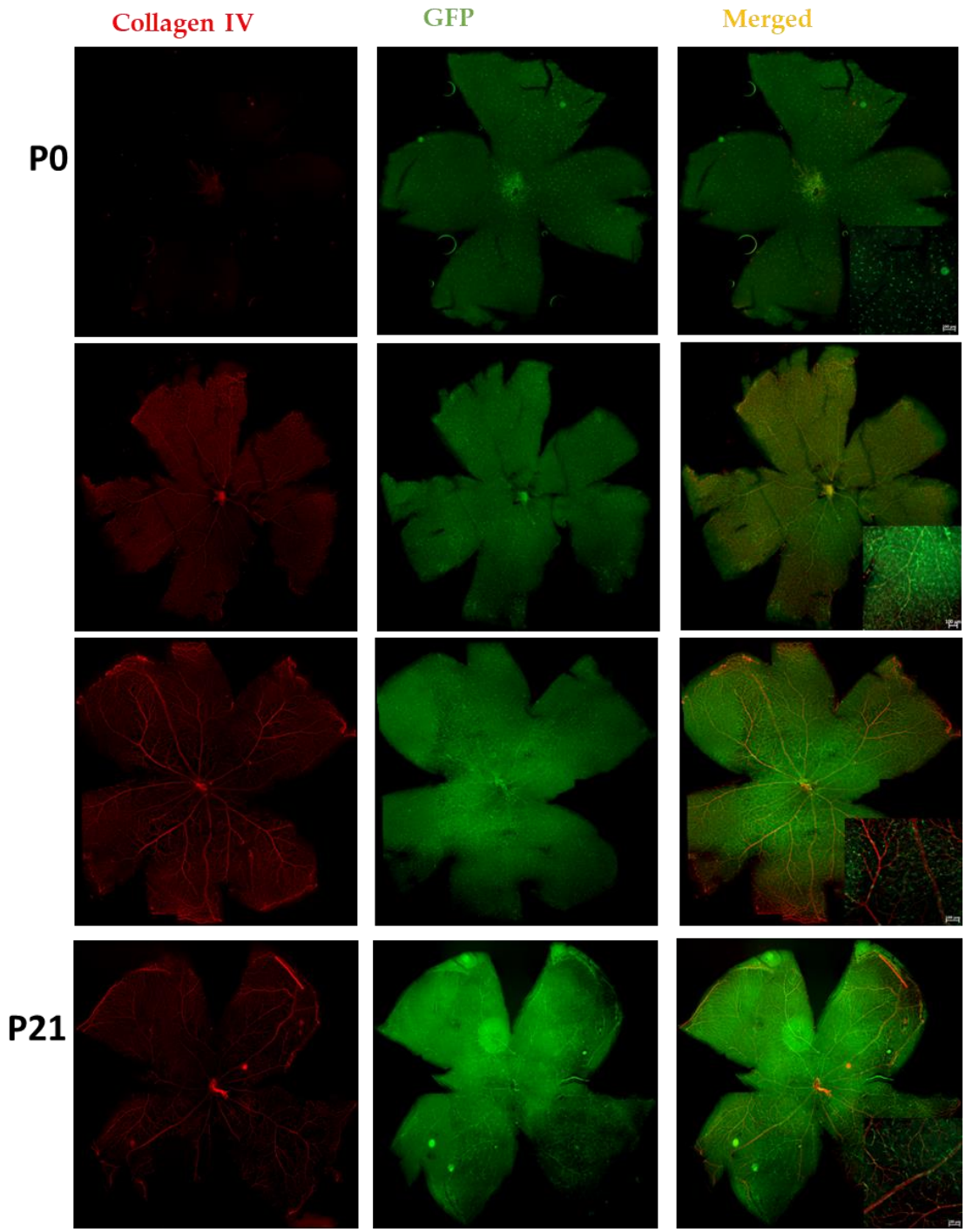

Collagen IV
GFP
Merged
P0
P21

Figure S1: Distribution of hematopoietic cells in retina development. Representative images showing the distribution of GFP+ hematopoietic cells recruited into the retina from P0 to P21. The recruited cells are distributed over the entire retina as early as P0 with no preference for the central or peripheral retina. Inserts are magnified images showing the uniform distribution at each time point.

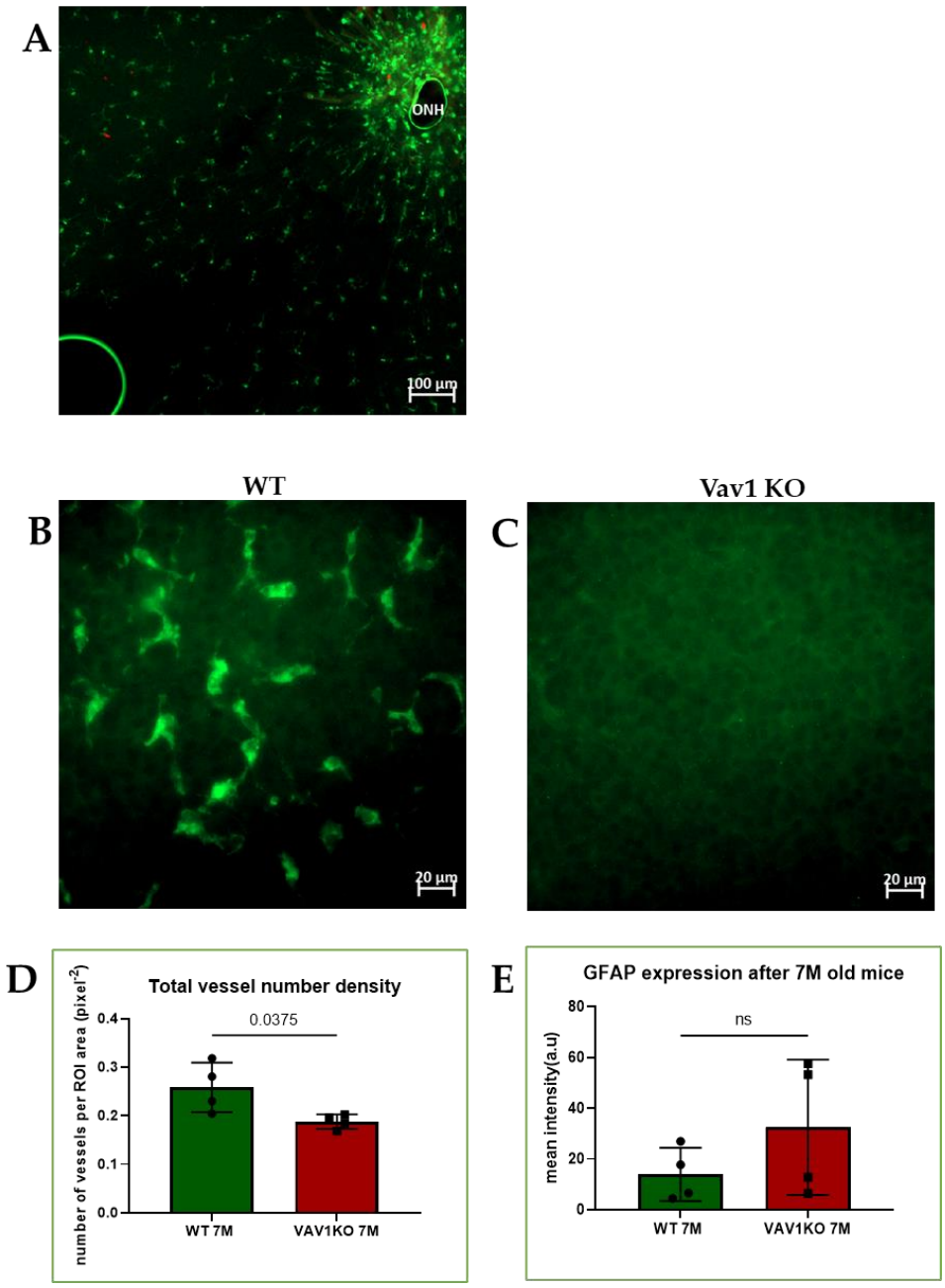


Figure S2: Hematopoietic cells play a role in vasculogenesis in retina development. A: Representative image showing the recruitment of GFP+ hematopoietic cells the retina at P0. The recruited cells mainly enter the retina posteriorly at the ONH and are distributed over the entire retina. (B) Representative images showing the expression of Vav1 in WT and (C)Vav1 KO retinas. Retinal flat mounts of wild-type (WT) and Vav1 KO mice were stained with rabbit anti-Vav1 (Cell Signalling Technology, Cat# 2502S). Vav1 KO retinas are devoid of Vav1+ hematopoietic cells. C: Bar graphs showing quantification of vascular density and GFAP expression in older (7-month-old wild-type and Vav1 KO mice. ONH: optic nerve head

PRESERVATION OF HEMATOPOIESIS IN THE CALVARIAL MARROW IN TYPE 2 DIABETIC MICE

by

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ABSTRACT

Hematopoiesis is the machinery responsible for the generation and renewal of blood cells in the body. Hematopoietic stem and progenitor cells exist in specialized microenvironments, called niches, inside the bone marrow of the long and flat bones throughout the body. These cells self-renew and differentiate upon stimulation to give rise to the mature myeloid and lymphoid cells that populate the blood. Much like other tissues, the bone marrow suffers diabetic end-organ damage, including aberrant adipogenesis, mobilopathy and microangiopathy. However, the impact of diabetes on hematopoiesis and on the bone marrow niches at different sites has not been studied. We show that the impact of diabetes is different in the calvarial marrow compartment compared to long bones. The calvarium undergoes slower bone deterioration, reduced buildup of fat content and less vascular degeneration compared to the tibia during chronic type 2 diabetes. Cumulatively, this results in a relative preservation of hematopoietic stem and progenitor cell function and an increase in erythroid lineage cells in the calvarial marrow during this period of chronic metabolic insult.

Keywords: Hematopoiesis, calvarium, long bones, tibia, relative preservation, vascular degeneration

INTRODUCTION

Diabetes is a complex, heterogenous metabolic disorder characterized by chronic hyperglycemia, hyperinsulinemia and dyslipidemia [171-177]. Like the eye, kidney and heart among other organs, current literature recognizes the bone marrow as another target of diabetic end-organ damage [99]. Diabetes alters the composition of the bone marrow microenvironment and its ultimate function of generating blood cells [99, 220]. Diabetes-associated fat accumulation, chronic inflammation, neuropathy and microangiopathy [178, 185-187, 220] impairs the mobilization of bone marrow reparative cells and skews hematopoiesis towards the myeloid lineage [191, 202, 204, 221]. Diabetic bone marrow-derived cells have a reduced ability to proliferate [203], are aberrantly localized in the niche [188] and generate more proinflammatory cells [204]. However, the impact of diabetes on different bone marrow sites has not been studied. It is not known whether diabetes homogenously affects the bone marrow in the calvarium and long bones or if one compartment of bone marrow is more resistant to diabetic damage than another. In this study, we show that the calvarial marrow undergoes slower bone deterioration, slower buildup of fat content and slower vascular degeneration compared to the tibia during chronic type 2 diabetes. We observed a preservation of hematopoietic stem and progenitor cell function and an increase in erythroid lineage cells in the calvarial marrow during this period of chronic metabolic insult.

METHODS

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham (APN 21802) and adhered to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Visual Research. 10-week-old adult db/db mice (B6.BKS(D)-Leprdb/J, Jackson Laboratories, Bar Harbor, ME) and wild-type controls (C57BL/6J, Jackson Laboratories, Bar Harbor, ME) were housed in a standard laboratory environment and maintained on a 12-hour light–dark cycle. Mice were euthanized after 13-15 months of age for experiments.

Ex vivo micro-CT imaging

After euthanasia, bones were isolated and cleaned of external tissue. The calvarium of each animal was divided into two halves along the sagittal suture. For each animal, one half of the calvarium and one tibia was used for micro-CT imaging (the remaining half calvaria and tibias were used for immunohistochemistry). For micro-CT imaging, bones were fixed in 4% PFA at 40C overnight, then washed and transferred into PBS and imaged using a μCT scanner (MiLabs, Utrecht, The Netherlands). All bone images were acquired at ultra-focused magnification with the following parameters: tube voltage (50 Kv), tube current (0.24 Ma), step angle (0.25o), and with 75-ms exposure. All images were reconstructed using vendor software at 20μm/voxel resolution for analysis. Data was analyzed using 3D Slicer v4.8.1. For the calvaria, the whole volumes of the frontal and parietal regions were used for analysis and quantification. For the tibias, the

entire volume of bone 3.66mm from the top edge (which corresponds to the proximal trabecular region) was analyzed and quantified.

Flow cytometry

To ensure all tissues are treated in a similar manner, bone marrow cells were obtained by bone crushing. Briefly, animals were euthanized and the tibias and calvaria were carefully dissected from the legs and skull respectively. Bones were cut into pieces with surgical scissors and then gently crushed for up to 1 minute each with a mortar and pestle three times in 1ml FACS buffer. Crushed bones were filtered into a 50ml conical tube through a 40um strainer. Samples were then centrifuged at 350g for 5 minutes. Cell pellets were resuspended in 1ml FACS buffer and counted using a hemocytometer. The cells were then incubated with a cocktail of antibodies for 45 minutes at room temperature in the dark. The antibodies used included Fixable Viability Dye eFluor506 (Invitrogen, Cat# 65-0866-14). CD45-APC-Cy7 (Invitrogen, Cat#47-0451-82), Mouse Hematopoietic Cell Lineage-FITC (Invitrogen, Cat# 22-7770-72), Sca-1-BV645 (Invitrogen, Cat# 64-5981-82), TER119-eFluor450 (Invitrogen, Cat# 48-5921-82) and CD71-FITC (BD Biosciences, Cat# 553266). Cells were then washed with FACS buffer, resuspended and analyzed using BD FACSCelesta flow cytometer (BD Biosciences) and FlowJo™ v10.8 Software (BD Life Sciences).

Colony-forming unit (CFU) assay

CFU assay was modified as previously published [222, 223]. Briefly, bone marrow single cell suspensions were prepared from the calvarium and tibia as described above. Red blood cells were lysed with ACK lysis buffer (Gibco, Cat# A1049201) for 5

minutes on ice, washed and resuspended in FACS buffer. Cells were plated in MethoCult GF M3434 (STEMCELL Technologies, Cat# 03434) according to manufacturer's instructions and allowed to grow for 12 days. Colonies were identified and counted following manufacturer's instructions. Afterwards, all colonies were harvested by adding 2mL of FACs buffer to each dish and pipetting gently to break the colonies into single cells in suspension. Cells in medium were transferred into a15mL conical tube and an additional 5 mL of FACs buffer was added to ensure that the methylcellulose is completely diluted. Samples were then centrifuged at 350g for 10 minutes to pellet the cells and washed again to remove any remaining methylcellulose. Cells were resuspended in 1ml FACS buffer and counted using a hemocytometer.

Immunohistochemistry

Immunohistochemical procedures were modified according to previously published protocols [224, 225]. Isolated bone samples were fixed in 4% formaldehyde/12.5% picric acid solution in 0.1M phosphate-buffered saline (PBS, ph 6.9) overnight at 4^{O}C. After fixation, the samples were decalcified for up to 2 weeks in 0.5 M EDTA, changing the solution every day. After decalcification, the samples were processed for paraffin embedding and then sectioned at 10-μm thickness. Paraffin-embedded sections were treated with xylene for 5 minutes (X3), followed by 100% ethanol and 95% ethanol. After washing with distilled water, sections were subjected to epitope retrieval in sodium citrate buffer for 40 minutes, washed in PBS (X3) and permeabilized with 0.3% Triton X-100 before blocking in 4% horse serum at room temperature for 45 minutes. Samples were incubated in primary antibodies (1:200 rabbit anti-collagen IV, Abcam, Cat#19808) overnight followed by goat anti-rabbit secondary

antibody AlexaFluor 647 (Catalog # A-21244) for 2 hours at room temperature. After washing three times in PBS, sections were counterstained with DAPI (Invitrogen, Cat#D3571) for 10 minutes at room temperature, washed and mounted for imaging. Images were obtained with a 40X objective lens and quantified using ImageJ.

Nile Red staining

Decalcified bones were incubated in 30% sucrose in PBS for at least 48 hours at 4^{o}C, embedded in optimal cutting temperature (OCT) medium and immediately frozen on dry ice and stored at -80 °C until further processing. The sections were thawed at 37 °C for 15-30 min., washed in PBS and stained with 5 μg/mL of Nile Red solution at room temperature for 15 minutes. Sections were washed and counterstained with DAPI for 10 minutes, washed and mounted for imaging.

RESULTS

'Red Marrow' in the calvarium and tibia in diabetes

We used the db/db mouse model to investigate differences in the calvarial and tibial bone marrow compartments in response to chronic type 2 diabetes. By examining the two tissues ex-vivo, we observed that the calvarium of db/db mice contained more red marrow than age-matched control calvarium (Figure 1A). However, the tibia of db/db mice had less red marrow than the control tibia, suggesting increased accumulation of adipocytes in the tibia of db/db mice, particularly in the epiphysis and metaphysis of the tibia (Figure 1B), but not in the calvarium.

Trabecular thickness and bone mineral density

Bone provides the structural environment for the bone marrow and osteoblasts and osteoclasts influence hematopoiesis [82]. Using microCT imaging, we examined the bone mineral density and trabecular thickness of calvarial and tibial bones of diabetic and control mice. As shown in Figure 2, an increase in trabecular thickness was observed in the frontal calvarium (but not parietal, Fig. 2C,D) of db/db mice compared to controls. In contrast, the trabecular thickness of db/db tibia was reduced (Fig 2. E). There was a trend towards increased bone mineral density in the calvarium (Fig. 2C,D) and a decrease in bone mineral density in the tibia of db/db mice (Fig. E).

Hematopoietic cells in the calvarium and tibia in diabetes

Using flow cytometry, we examined the composition of hematopoietic cells in the two distinct locations (Figures 3 and 4). The calvarial marrow in db/db mice had higher cellularity (cell density) compared to controls. In contrast, the cellularity of the tibia was

reduced in db/db mice (Figure 3A). Next, we tested if the HSPCs proportion in the calvarium was different from that of the tibia in type 2 diabetic mice. While the proportion of HSPCs in the tibia did not differ between diabetic mice and controls, a higher proportion of HSPCs were seen in the calvarium of db/db mice compared to controls (Figure 3C), suggesting a relative preservation of HSPCs in the calvarium in chronic diabetes. The proportion of mature hematopoietic cells ($CD45^+$) was reduced in both tibia and calvarium of db/db mice (Figure 4) compared to controls. However, the reduction was less in the calvarium (52.78% ± 4.22 vs 32.13% ± 9.25 for controls and db/db, respectively) than in the tibia (43.43% ± 3.73 vs 13.39% ± 3.01 for controls and dbdb, respectively).

Since the tissues showed different amounts of red marrow ex vivo (Figure 1), we investigated whether there were any differences in the proportion of erythroid lineage cells in the marrows. As shown in Figure 4, we observed that the db/db calvarium contained significantly more $TER119^+CD71^-$ mature erythrocytes while the db/db tibia showed a trend towards reduced $TER119^+CD71^-$ mature erythrocytes. Also, the db/db tibia showed a significantly reduced proportion of $TER119^+CD71^+$ erythroid precursors while the db/db calvarium showed a trend towards increased proportion of erythroid precursors, suggesting increased erythropoiesis in the calvarial marrow but not in the tibia in db/db mice.

Functional hematopoiesis in the calvarium and tibia in diabetes

We next examined the proliferation potential of bone marrow-derived cells from the calvarium and tibia using the colony-forming assay. Bone marrow cells isolated from either the calvarium or tibia were plated and allowed to multiply and expand for 12 days

in vitro. As shown in Figure 5, we observed a trend towards an increased total number of colonies formed by cells isolated from the db/db calvarium compared to controls. This was not the case for the cells isolated from the tibia. When the colonies were harvested and the cells counted, we observed that bone marrow cells from the calvarium of db/db mice gave rise to colonies with higher cellularity (Fig. 5A) compared to controls but not the tibial cells (Fig. 5B). Collectively, this suggests that the calvarium preserves the function and proliferative potential of hematopoietic cells compared to the tibia in type 2 diabetes.

Vascular density and fat content of the calvarium and tibia in diabetes

We next examined the cellularity, vascular density and adipocyte content of the distinct bone marrow locations in chronic type 2 diabetes (Figures 6 and 7) using collagen IV to label the bone marrow vasculature, nile red to stain lipids and DAPI to identify cells in bone marrow cross sections. Similar to what we observed with single cell suspensions in Figure 3, we observed that the db/db calvarial marrow had significantly higher cellularity compared to controls, while the db/db tibia had significantly reduced cellularity (Figure 6B). In addition, both the db/db calvarium and db/db tibia had significantly reduced vascular density compared to controls (Figure 6D). However, the reduction in vascular density in the calvarium was less (5.7au ± 1.9 vs 3.2au ± 0.9 for controls vs dbdb, respectively) than the reduction observed in the tibia (13.6au ± 2.7 vs 3.4au ± 3.1 for controls vs dbdb, respectively). This suggested a slower vascular degeneration in the calvarial marrow compared to the tibia in the dbdb mice. Previously, type 2 diabetes has been associated with increased adipogenesis in the bone marrow of long bones [187, 210, 213]. Thus, we investigated whether there were any differences in

fat/lipid accumulation between the two distinct marrow locations using nile red staining. As shown in Figure 7, we observed higher nile red fluorescence intensity in db/db tibia compared to controls but not in the dbdb calvarium, indicating a higher lipid accumulation in the db/db tibia than the calvarium.

DISCUSSION

In this study, we show that the calvarial and tibial marrows respond differently to chronic type 2 diabetic insults. The calvarial marrow shows increased trabecular thickness and cellularity and a preserved proportions and function of hematopoietic stem and progenitor cells compared to the tibia. In addition, the calvarial marrow accumulates less fats and has a slower rate of diabetes-associated vascular degeneration. Our data supports a previously unknown facet of diabetes-induced bone marrow pathology in which the calvarial marrow undergoes slower remodeling and shows better preservation of hematopoiesis.

The bone marrow, a gelatinous tissue found in long and flat bones in the body, houses essential proinflammatory, anti-inflammatory and reparative cells critical to the immune response to disease and injury. It is well known that the metabolic insults of diabetes disrupts the balance between inflammatory and reparative mechanisms in the bone marrow [99, 178-180], skewing the generation of cells towards the inflammatory phenotype in long bones [204, 208]. In addition, chronic diabetes affects the bones that house the marrow. Diabetic individuals have increased risk of osteoporosis due to increased bone loss, reduced bone synthesis or both [226-228]. We sought to understand if the calvarial and tibial bones are affected differently by chronic type 2 diabetes. Using microCT imaging, we observed an increase in the trabecular thickness of the db/db calvarium while that of the db/db tibia was reduced compared to controls. Diabetes alters the balance between osteoblast and osteoclast activity, whereby elevated osteoclast numbers and activity leads to increased bone resorption while reduced osteoblast numbers leading to poor bone formation and quality [229, 230]. Type 2 diabetes leads to

bigger cavities in the trabecular network and reduced osteoblast recruitment in the long bones [231, 232]. Thus, the increase in trabecular thickness in the frontal calvarium compared to controls (Fig 2) and increasing cellularity (Fig 3) could be due to an increase in osteoblast number and activity. Suppression of osteoclastic activity or increase in osteoblast activity in the calvarium compared to the long bones of the tibia could similarly result in increased trabecular thickness. Osteoblasts are an essential component of the hematopoietic stem cell niche and an increase in osteoblast number and activity has been shown to increase HSC function [73, 130]. Type 2 diabetes is known to adversely impact bone mass but not as dramatic as type 1 diabetes; however, bone quality is still impaired [228, 233]. This could account for the trends towards increasing bone mineral density (BMD) of the calvarium of dbdb mice vs controls and decreasing BMD of the tibia in dbdb vs controls observed in our study that did not reach statistical significance. Our data suggests a potentially slower rate of bone resorption or increased bone formation in the calvarium compared to the tibia of type 2 diabetic mice, facilitating a microenvironment more conducive to maintaining hematopoiesis in the calvarium than in the tibia.

Type 2 diabetes leads to the accumulation of adipocytes in the bone marrow and affects hematopoiesis [187, 234]. Long-standing diabetes leads to increased adipocytes by shifting the differentiation of bone marrow mesenchymal stem cells towards adipocytic lineage, thereby suppressing the differentiation of osteoblasts and impairing hematopoietic stem cell function [212-216]. In our study, we observed ex-vivo that the db/db calvarial marrow contained more red marrow than controls (Fig 1). This was evident in the frontal, parietal and occipital regions of the calvarium. However, the db/db

tibia was observed to contain less red marrow. We employed nile red staining, a fluorescent labeling technique specific for lipid droplets [235] to explore the accumulation of lipids in the bone marrow in diabetes (Fig 7). We observed an increase in nile red fluorescence in db/db tibias compared to controls but not in db/db calvarium. This suggests increased adipogenesis in the tibias of db/db mice while the calvarial marrow was relatively spared. The increased adipogenesis likely suppresses the differentiation of mesenchymal stem cells into non-adipocytic phenotypes critical to the maintenance of the bone marrow niche [217] and this adversely affects hematopoiesis in the tibia compared to the calvarium.

The impact of diabetes on hematopoietic cells has been well documented, including impaired endothelial progenitor cell mobilization, impaired hematopoietic stem cell proliferation and myelopoiesis [179, 191, 194, 204, 220, 236]. We sought to pursue the ex vivo observations that the calvarium compartment contained more "red" marrow in the diabetic mice compared to age matched controls. When we analyzed the cellularity of both compartments by flow cytometry (Fig 4) and immunohistochemistry (Fig 6), we observed that the db/db calvarial marrow had more cells compared to controls, while the cellularity of the db/db tibia was reduced compared to age-matched controls. In addition, the proportion of erythroid lineage cells were higher in the db/db calvarium compared to controls but was reduced in the db/db tibia. However, the proportion of mature hematopoietic cells ($CD45^+$), which makes up the majority of hematopoietic cells in the bone marrow, was significantly reduced in both locations. This suggests that the increase in cellularity of the db/db calvarium could in part be due to expansion of non-hematopoietic and stromal cells, such as osteoblasts responsible for bone formation, or

increased erythropoiesis, or both, in the db/db calvarial marrow. We also assessed the proportion and function of HSPCs in the distinct bone marrow regions. We observed that while the proportion of HSPCs in the tibia remained unchanged between dbdb and controls, the dbdb calvarium had significantly higher proportion of HSPCs. When we tested the proliferation potential of bone marrow-derived cells from the two locations, we observed that cells from the dbdb calvarium formed colonies of higher cellularity compared to controls (Fig 5). Studies have reported a reduction in HSPCs in the long bones of type 2 diabetic individuals [210, 220] and that diabetic HSPCs are dysfunctional [237, 238]. Thus, our data suggests that the calvarial marrow relatively preserves HSPCs and hematopoiesis in type 2 diabetes. It has been shown that the calvarial marrow is directly connected to the cerebrospinal fluid (CSF) through microscopic channels that cross the inner skull cortex and directly connect the skull bone marrow cavities with the dura of the brain [64, 123]. The calvarial marrow receives CSF through perivascular spaces traversing the dura [124]. CSF is a source of neurotrophic and growth factors from the central nervous system, including oxysterols (endogenous Liver X receptor (LXR) ligands) [239-241]. Previously, we showed that LXR activation with N, N-dimethyl-3β-hydroxy-cholenamide (DMHCA) is sufficient to correct BM dysfunction in db/db mice [222]. DMHCA restored cholesterol homeostasis and rejuvenated membrane fluidity in bone marrow cells. Using single-cell RNA sequencing on lineage-sca1+c-Kit+ (LSK) hematopoietic stem cells (HSCs) from untreated and DMHCA-treated db/db mice, we showed that DMHCA reduced myeloidosis, increased myeloid angiogenic cells (MACs; vascular reparative cells) and increased erythrocyte progenitors. We show in this study that the calvarium maintains erythropoiesis and preserves HSPCs better than the tibial

compartments and this may be due to the presence of central nervous system-derived endogenous LXR agonists in the bone marrow of the calvarium but not of the tibia.

Diabetes causes microangiopathy in peripheral tissues including the bone marrow [210], characterized by endothelial dysfunction, increased vascular permeability [178, 210], remodeling and loss of capillaries [187]. We examined the vasculature in the calvarial and tibial marrows of db/db mice and controls to determine if the apparent preservation of hematopoiesis in the calvarial marrow is associated with improved vasculature. We observed that both db/db calvaria and db/db tibias had reduced vascular density compared to controls (Fig 6). However, the reduction in vascular density in the db/db calvarium was approximately four times less than the reduction in the tibia. This suggests a slower rate of diabetes-induced vascular degeneration in db/db calvarium compared to db/db tibia. Beneficial growth factors provided by the CSF in the calvarial marrow could be a contributing factor in delaying vascular degeneration in this site.

CONCLUSIONS AND FUTURE DIRECTIONS

Our study shows that the calvarial marrow undergoes slower deterioration in type 2 diabetes compared to the tibia. In diabetes, the calvarium preserves the function and proliferative potential of hematopoietic cells longer than the tibia. Given that this phenomenon has been observed in other flat bones such as the iliac crest in humans [242], the more easily accessible calvarium provides an excellent opportunity to explore further the inherent mechanisms that protects the marrow in flat bones. In this regard, the impact of cerebrospinal fluid-derived endogenous LXR ligands and growth factors may be the mechanism that protects the calvarium from the damaging effects of chronic type 2 diabetes.

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REFERENCES

1. Dobbs, R., et al., *Glucagon: role in the hyperglycemia of diabetes mellitus.* Science, 1975. **187**(4176): p. 544-547.

2. Stern, M.P. and S.M. Haffner, *Body fat distribution and hyperinsulinemia as risk factors for diabetes and cardiovascular disease.* Arteriosclerosis: An Official Journal of the American Heart Association, Inc., 1986. **6**(2): p. 123-130.

3. Magaji, V. and J.M. Johnston, *Inpatient management of hyperglycemia and diabetes.* Clinical Diabetes, 2011. **29**(1): p. 3-9.

4. Ceriello, A., *Postprandial hyperglycemia and diabetes complications: is it time to treat?* Diabetes, 2005. **54**(1): p. 1-7.

5. Kulkarni, R.N., et al., *Impact of genetic background on development of hyperinsulinemia and diabetes in insulin receptor/insulin receptor substrate-1 double heterozygous mice.* Diabetes, 2003. **52**(6): p. 1528-1534.

6. Solano, M.P. and R.B. Goldberg, *Management of dyslipidemia in diabetes.* Cardiology in review, 2006. **14**(3): p. 125-135.

7. Mooradian, A.D., *Dyslipidemia in type 2 diabetes mellitus.* Nature Reviews Endocrinology, 2009. **5**(3): p. 150-159.

8. Fadini, G.P., et al., *Concise review: diabetes, the bone marrow niche, and impaired vascular regeneration.* Stem cells translational medicine, 2014. **3**(8): p. 949-957.

9. Kojima, H., J. Kim, and L. Chan, *Emerging roles of hematopoietic cells in the pathobiology of diabetic complications.* Trends in Endocrinology & Metabolism, 2014. **25**(4): p. 178-187.

10. Fadini, G.P., S. Ciciliot, and M. Albiero, *Concise review: perspectives and clinical implications of bone marrow and circulating stem cell defects in diabetes.* Stem Cells, 2017. **35**(1): p. 106-116.

11. Mangialardi, G., et al., *Diabetes causes bone marrow endothelial barrier dysfunction by activation of the RhoA–Rho-associated kinase signaling pathway.* Arteriosclerosis, thrombosis, and vascular biology, 2013. **33**(3): p. 555-564.

12. Albiero, M., et al., *Diabetes causes bone marrow autonomic neuropathy and impairs stem cell mobilization via dysregulated p66Shc and Sirt1.* Diabetes, 2014. **63**(4): p. 1353-1365.

13. Busik, J.V., et al., *Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock.* Journal of Experimental Medicine, 2009. **206**(13): p. 2897-2906.

14. Albiero, M., et al., *Diabetes-associated myelopoiesis drives stem cell mobilopathy through an OSM-p66Shc signaling pathway.* Diabetes, 2019. **68**(6): p. 1303-1314.

15. Nagareddy, P.R., et al., *Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis.* Cell metabolism, 2013. **17**(5): p. 695-708.

16. Tepper, O.M., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-2786.

17. Fadini, G.P., et al., *Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus.* Journal of the American College of Cardiology, 2005. **45**(9): p. 1449-1457.

18. Hazra, S., et al., *Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model.* Diabetologia, 2013. **56**(3): p. 644-653.

19. Ferraro, F., et al., *Diabetes impairs hematopoietic stem cell mobilization by altering niche function.* Science translational medicine, 2011. **3**(104): p. 104ra101-104ra101.

20. Vieira, C.P., et al., *Selective LXR agonist DMHCA corrects retinal and bone marrow dysfunction in type 2 diabetes.* JCI insight, 2020. **5**(13).

21. Duan, Y., et al., *Loss of angiotensin-converting enzyme 2 exacerbates diabetic retinopathy by promoting bone marrow dysfunction.* Stem Cells, 2018. **36**(9): p. 1430-1440.

22. Chartier, S.R., et al., *The changing sensory and sympathetic innervation of the young, adult and aging mouse femur.* Neuroscience, 2018. **387**: p. 178-190.

23. Isidro, R.A., et al., *Double immunofluorescent staining of rat macrophages in formalin-fixed paraffin-embedded tissue using two monoclonal mouse antibodies.* Histochemistry and cell biology, 2015. **144**(6): p. 613-621.

24. Kiel, M.J. and S.J. Morrison, *Maintaining hematopoietic stem cells in the vascular niche.* Immunity, 2006. **25**(6): p. 862-864.

25. Spinetti, G., et al., *Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microRNA-155/FOXO3a signaling pathway.* Circulation research, 2013. **112**(3): p. 510-522.

26. Botolin, S. and L.R. McCabe, *Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice.* Endocrinology, 2007. **148**(1): p. 198-205.

27. Santopaolo, M., et al., *Bone marrow as a target and accomplice of vascular complications in diabetes.* Diabetes/Metabolism Research and Reviews, 2020. **36**: p. e3240.

28. Oikawa, A., et al., *Diabetes mellitus induces bone marrow microangiopathy.* Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(3): p. 498-508.

29. Nagareddy, P.R., et al., *Adipose tissue macrophages promote myelopoiesis and monocytosis in obesity.* Cell metabolism, 2014. **19**(5): p. 821-835.

30. Lin, Y., et al., *Activation of osteoblast ferroptosis via the METTL3/ASK1-p38 signaling pathway in high glucose and high fat (HGHF)-induced diabetic bone loss.* The FASEB Journal, 2022. **36**(3): p. e22147.

31. Mohsin, S., et al., *An update on therapies for the treatment of diabetes-induced osteoporosis.* Expert Opinion on Biological Therapy, 2019. **19**(9): p. 937-948.

32. Hamann, C., et al., *Bone, sweet bone—osteoporotic fractures in diabetes mellitus.* Nature Reviews Endocrinology, 2012. **8**(5): p. 297-305.

33. Catalfamo, D.L., et al., *Hyperglycemia induced and intrinsic alterations in type 2 diabetes-derived osteoclast function.* Oral diseases, 2013. **19**(3): p. 303-312.

34. Yang, M., et al., *Tubeimoside I suppresses diabetes-induced bone loss in rats, osteoclast formation, and RANKL-induced nuclear factor-κB pathway.* International Immunopharmacology, 2020. **80**: p. 106202.

35. Pritchard, J.M., et al., *Association of larger holes in the trabecular bone at the distal radius in postmenopausal women with type 2 diabetes mellitus compared to controls.* Arthritis care & research, 2012. **64**(1): p. 83-91.

36. Rathinavelu, S., C. Guidry-Elizondo, and J. Banu, *Molecular modulation of osteoblasts and osteoclasts in type 2 diabetes.* Journal of diabetes research, 2018. **2018**.

37. Calvi, L., et al., *Osteoblastic cells regulate the haematopoietic stem cell niche.* Nature, 2003. **425**(6960): p. 841-846.

38. Zhang, J., et al., *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature, 2003. **425**(6960): p. 836-841.

39. Schwartz, A.V. and D.E. Sellmeyer, *Women, type 2 diabetes, and fracture risk.* Current diabetes reports, 2004. **4**(5): p. 364-369.

40. Ferland-McCollough, D., et al., *MCP-1 feedback loop between adipocytes and mesenchymal stromal cells causes fat accumulation and contributes to hematopoietic stem cell rarefaction in the bone marrow of patients with diabetes.* Diabetes, 2018. **67**(7): p. 1380-1394.

41. Keats, E. and Z.A. Khan, *Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose.* PloS one, 2012. **7**(6): p. e38752.

42. Keats, E.C., et al., *Switch from canonical to noncanonical Wnt signaling mediates high glucose-induced adipogenesis.* Stem Cells, 2014. **32**(6): p. 1649-1660.

43. Kim, T.Y. and A.L. Schafer, *Diabetes and bone marrow adiposity.* Current osteoporosis reports, 2016. **14**(6): p. 337-344.

44. Botolin, S., et al., *Increased bone adiposity and peroxisomal proliferator-activated receptor-γ2 expression in type I diabetic mice.* Endocrinology, 2005. **146**(8): p. 3622-3631.

45. Greenspan, P., E.P. Mayer, and S.D. Fowler, *Nile red: a selective fluorescent stain for intracellular lipid droplets.* The Journal of cell biology, 1985. **100**(3): p. 965-973.

46. Piccinin, M.A. and Z.A. Khan, *Pathophysiological role of enhanced bone marrow adipogenesis in diabetic complications.* Adipocyte, 2014. **3**(4): p. 263-272.

47. Loomans, C.J., et al., *Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes.* Diabetes, 2004. **53**(1): p. 195-199.

48. Egan, C., et al., *Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes.* Diabetologia, 2008. **51**(7): p. 1296-1305.

49. Bhatwadekar, A.D., et al., *Hematopoietic stem/progenitor involvement in retinal microvascular repair during diabetes: Implications for bone marrow rejuvenation.* Vision research, 2017. **139**: p. 211-220.

50. Chakravarthy, H., et al., *Role of acid sphingomyelinase in shifting the balance between proinflammatory and reparative bone marrow cells in diabetic retinopathy.* Stem cells, 2016. **34**(4): p. 972-983.

51. Poller, W.C., et al., *Brain motor and fear circuits regulate leukocytes during acute stress.* Nature, 2022: p. 1-3.

52. Herisson, F., et al., *Direct vascular channels connect skull bone marrow and the brain surface enabling myeloid cell migration.* Nature neuroscience, 2018. **21**(9): p. 1209-1217.

53. Pulous, F.E., et al., *Cerebrospinal fluid can exit into the skull bone marrow and instruct cranial hematopoiesis in mice with bacterial meningitis.* Nature Neuroscience, 2022. **25**(5): p. 567-576.

54. Theofilopoulos, S. and E. Arenas, *Liver X receptors and cholesterol metabolism: role in ventral midbrain development and neurodegeneration.* F1000prime Reports, 2015. **7**.

55. Mills III, W.A., M.A. Coburn, and U.B. Eyo, *The emergence of the calvarial hematopoietic niche in health and disease.* Immunological Reviews, 2022.

56. Bjurstrom, M.F., S.E. Giron, and C.A. Griffis, *Cerebrospinal fluid cytokines and neurotrophic factors in human chronic pain populations: a comprehensive review.* Pain Practice, 2016. **16**(2): p. 183-203.

57. Eventov, I., et al., *Osteopenia, hematopoiesis, and bone remodelling in iliac crest and femoral biopsies: a prospective study of 102 cases of femoral neck fractures.* Bone, 1991. **12**(1): p. 1-6.

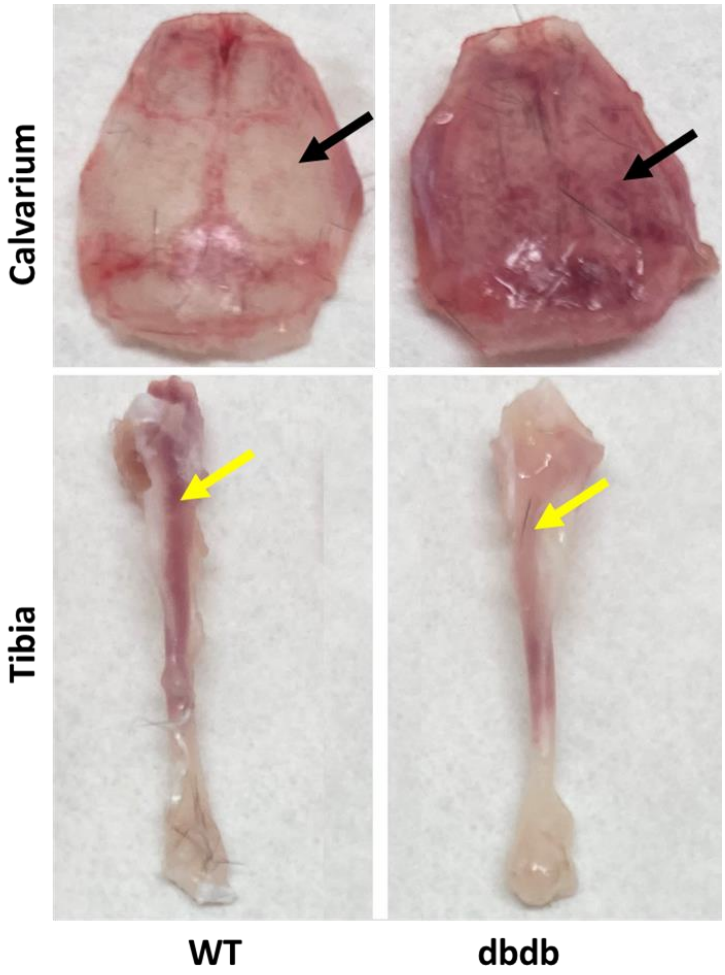


Figure 1. Ex-vivo observation of calvarial and tibial marrows in chronic type 2 diabetes. Photographs showing differences in bone marrow content between the calvarium and tibia in chronic type 2 diabetes. Db/db mice show more red marrow and less fat content in the calvarium than controls (A, black arrows). However, db/db tibias have less red marrow and more fat content than controls (B, yellow arrows).

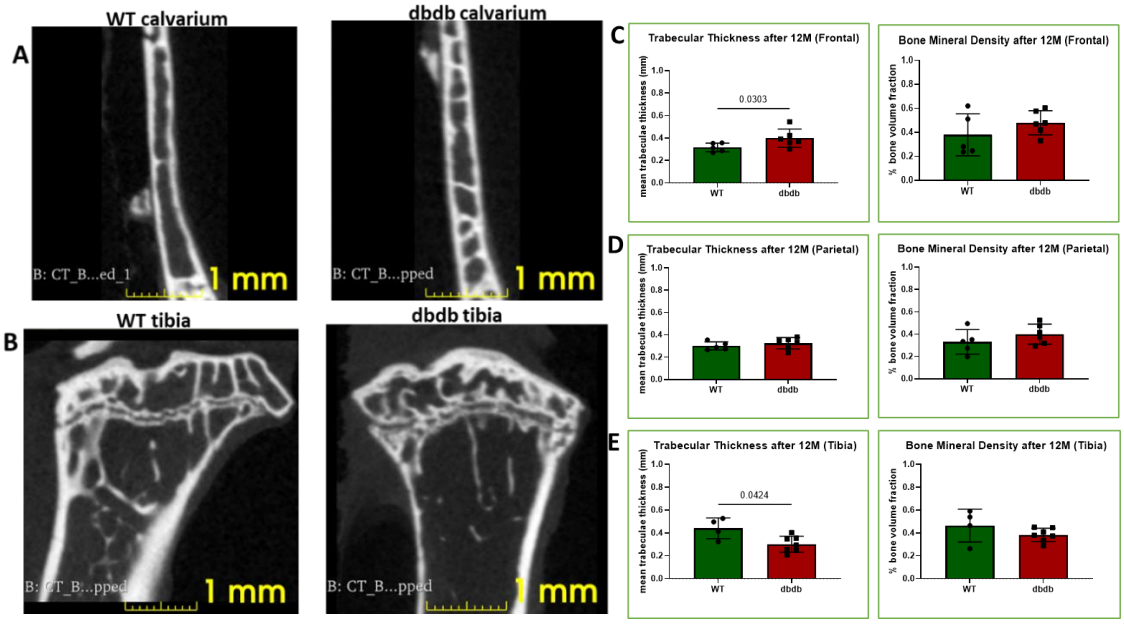


Figure 2. Bone mineral density and trabecular thickness of calvarium and tibia in db/db and controls. Representative images of microCT scans of the calvarium (A) and tibia (B) of control and db/db mice. Db/db mice have significantly increased trabecular thickness in the frontal calvarium (C) (but not parietal, D) compared to controls. However, the trabecular thickness in the tibia is reduced in dbdb mice (E).

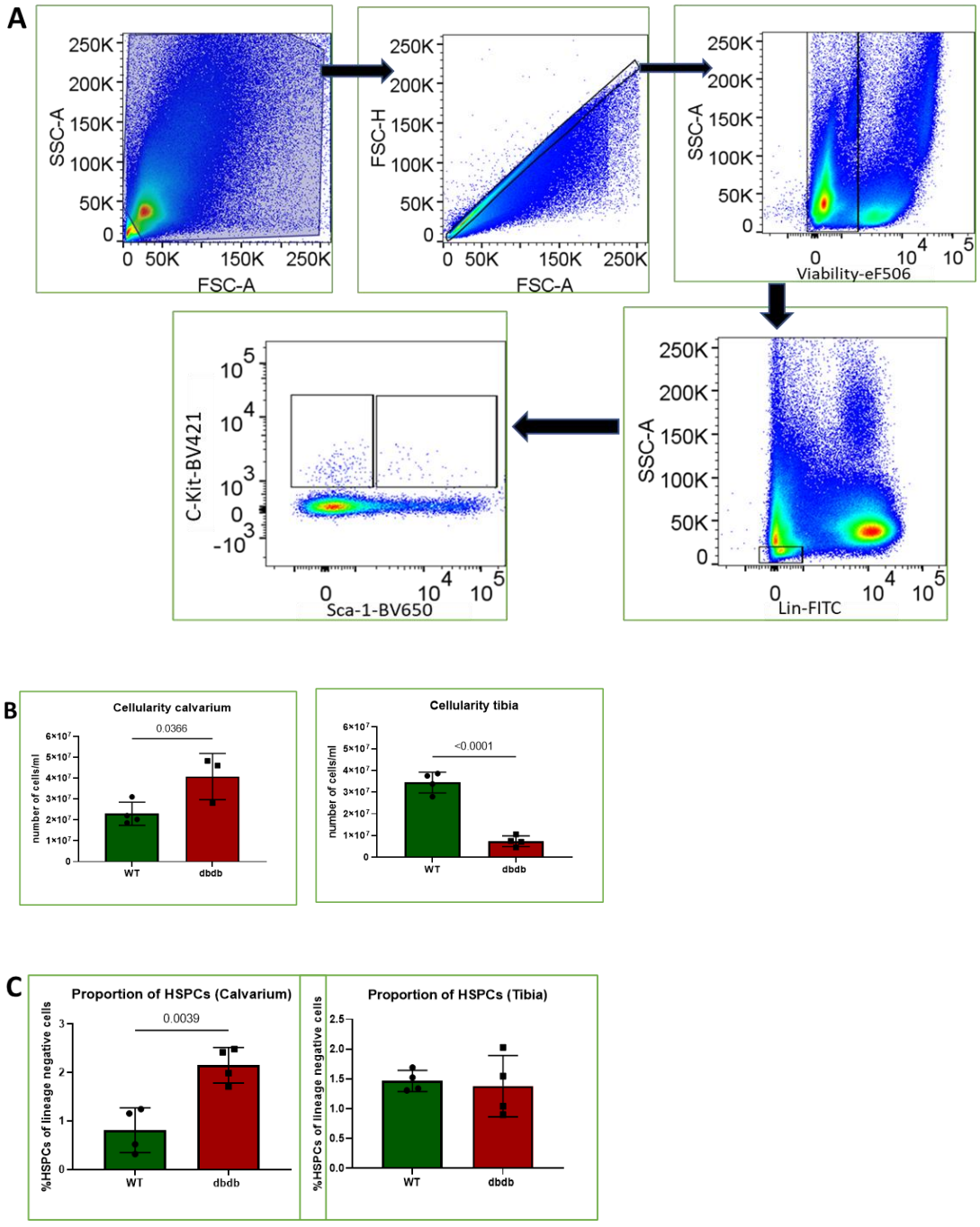
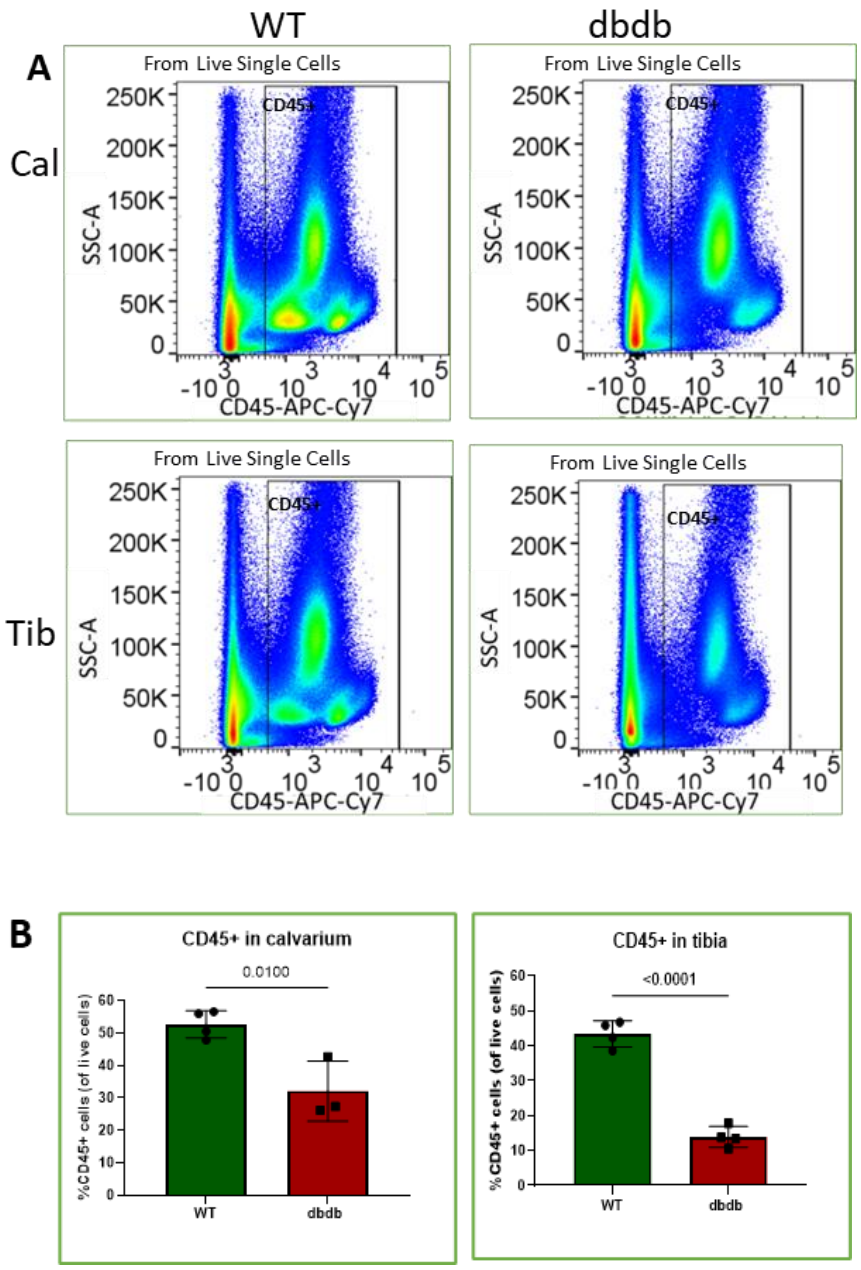
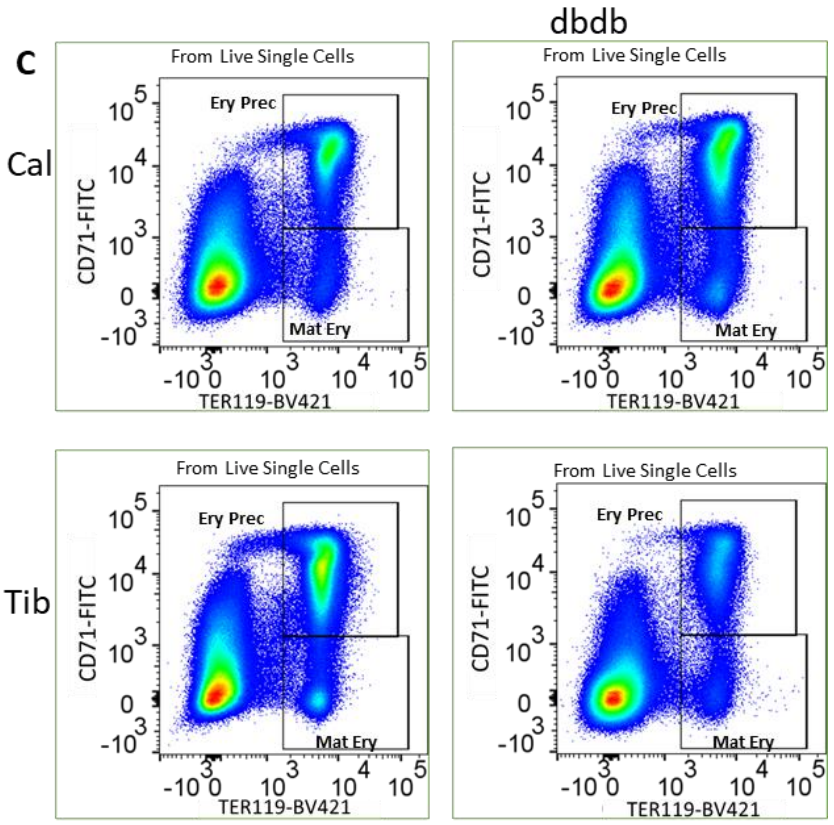


Figure 3. Flow cytometric characterization of hematopoietic stem and progenitor cells (HSPCs) in the calvarium and tibia of dbdb and control mice. A: Representative flow plots showing flow gating strategy for Lin-/Sca-1-/C-kit+ (LS-K) and Lin-/Sca-1+/C-kit+ (LSK) cells. LS-K and LSK cells were combined into HSPCs. B: Bar graphs showing quantification of cellularity (cell density) of calvarial and tibial marrows. C: Bar graphs showing quantification of the proportions of HSPCs in the two marrow sites.


WT
dbdb
A
Cal
Tib
From Live Single Cells
CD45+
SSC-A
CD45-APC-Cy7
250K
200K
150K
100K
50K
0
B
CD45+ in calvarium
0.0100
%CD45+ cells (of live cells)
WT
dbdb
CD45+ in tibia
<0.0001


C
dbdb
Cal
Tib
From Live Single Cells
Ery Prec
Mat Ery
CD71-FITC
TER119-BV421

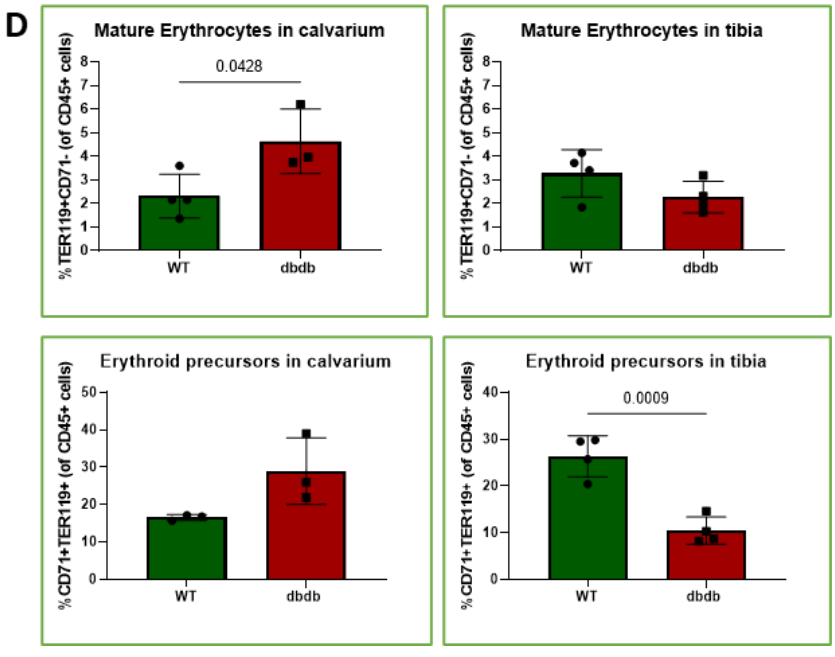

D
Mature Erythrocytes in calvarium
0.0428
% TER119+CD71- (of CD45+ cells)
WT
dbdb
Mature Erythrocytes in tibia
Erythroid precursors in calvarium
% CD71+TER119+ (of CD45+ cells)
Erythroid precursors in tibia
0.0009

Figure 4. Flow cytometric characterization of CD45+ cells and erythroid lineage cells in the calvarium and tibia of db/db and control mice. A: Representative flow plots showing flow gates for $C45^{+}$ cells from calvarium (Cal, top row) and tibia (Tib, bottow row). B: Bar graphs showing quantification from A. C: Representative flow plots showing flow gates for erythroid precursors (Ery Prec, CD71+TER119+) and mature erythrocytes (Mat Ery, CD71-TER119+) from calvarium (Cal, top row) and tibia (Tib, bottow row). D: Bar graphs showing quantification from C. Ery Prec: erythroid precursors; Mat Ery: mature erythrocytes

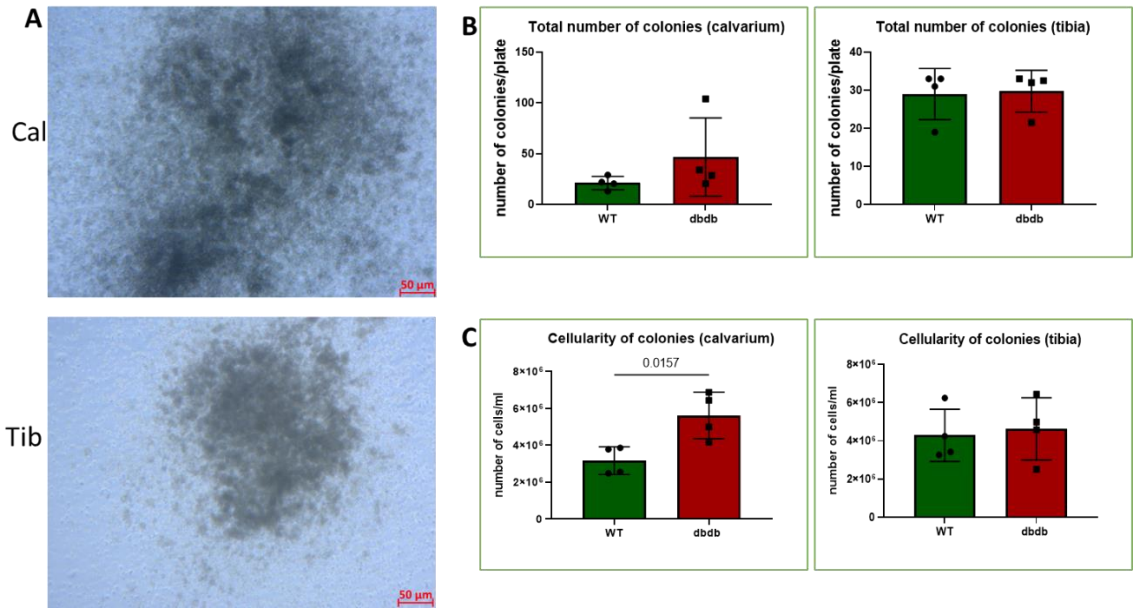
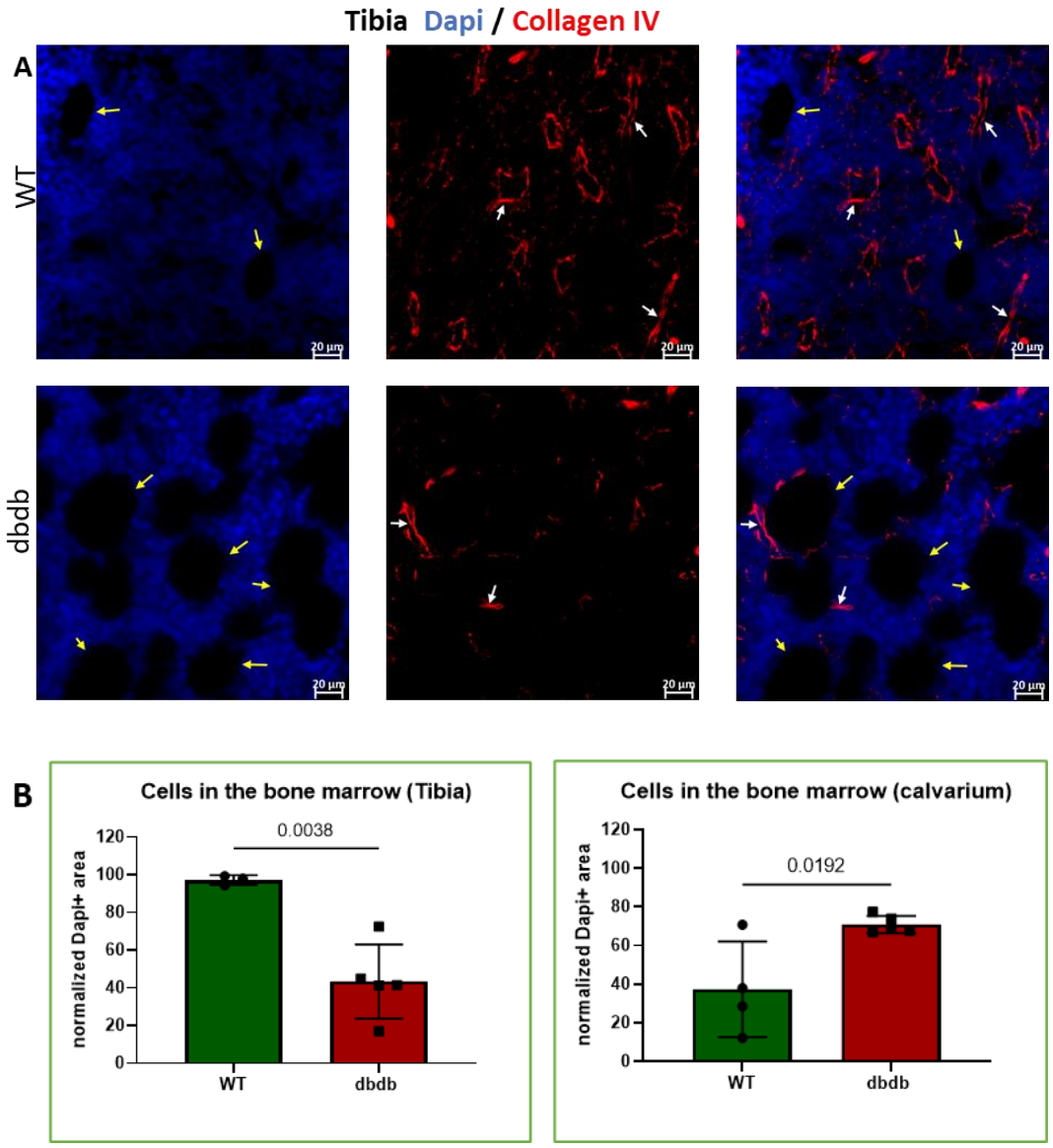


Figure 5. Colony-Forming Unit (CFU) assay for bone marrow-derived cells from the calvarium and tibia of db/db mice versus controls. A: Representative images of colonies formed by bone marrow-derived cells from the calvarium (Cal, top image) and tibia (Tib, bottom image). Bar graphs show the quantification of colonies (B) and single cells in suspension (C). After 12 days post-seeding, there is a trend towards increasing number of colonies from calvarial cells of db/db mice. Single cell suspension of the harvested colonies shows a significant increase in cellularity of colonies from db/db calvarium compared to controls, but not from tibial colonies.


Tibia Dapi / Collagen IV
A
WT
dbdb
20 µm
B
Cells in the bone marrow (Tibia)
normalized Dapi+ area
0.0038
120
100
80
60
40
20
0
WT
dbdb
Cells in the bone marrow (calvarium)
normalized Dapi+ area
0.0192
120
100
80
60
40
20
0
WT
dbdb

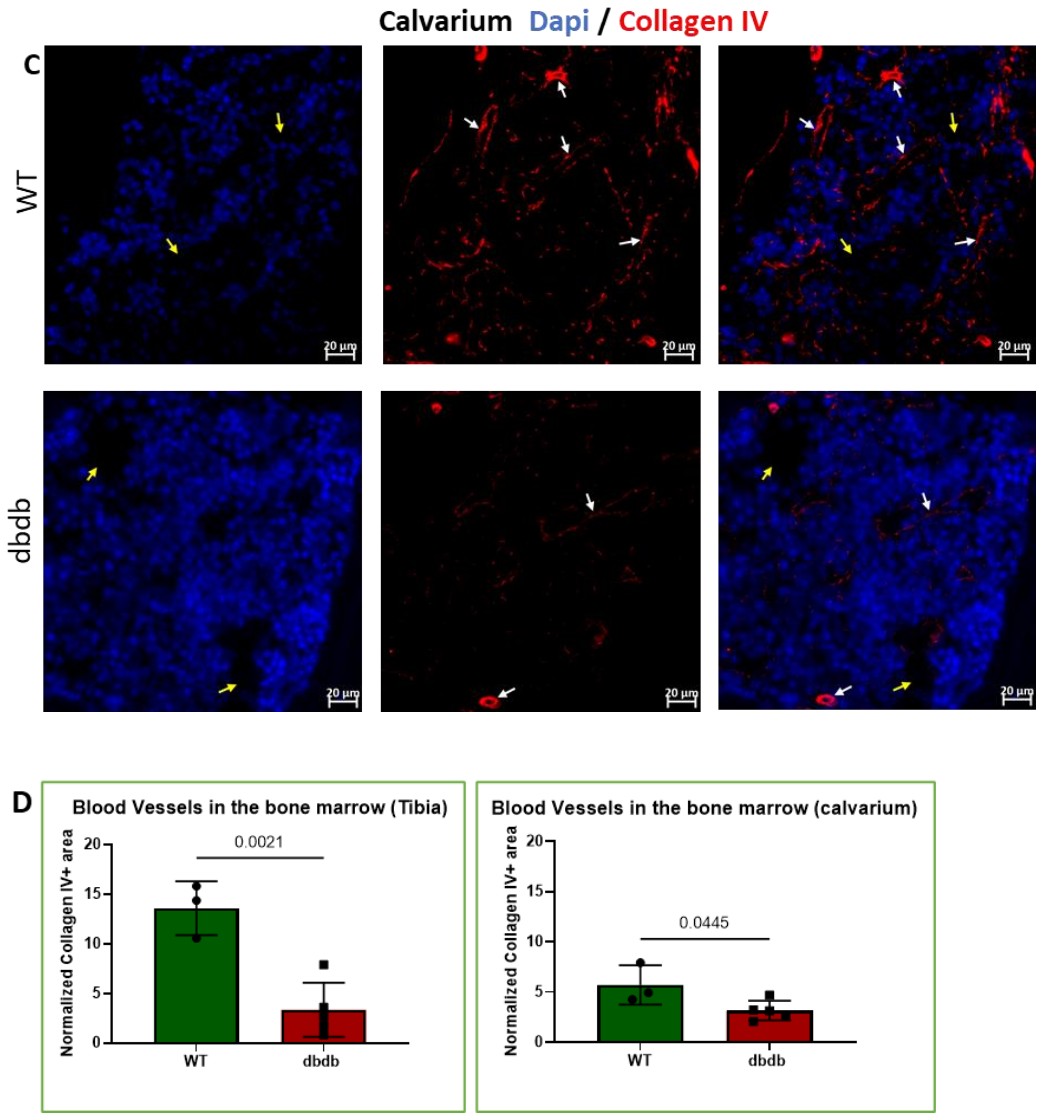


Figure 6. Cellularity and vascular density of calvarial and tibial marrows in dbdb vs controls. A,C: Representative images showing bone marrow cellularity (DAPI+) and blood vessels (collagen IV+). Dbdb calvarial marrow has increased number of cells (Dapi+) compared to controls while the number of cells in the tibia is reduced in dbdb mice (B). In both calvarium and tibia, the vascular densities are significantly reduced in dbdb mice (D). However, the vascular degeneration is worse in dbdb tibia. White arrows indicate collagen IV vessels, yellow arrows indicate 'empty' spaces filled by lipids/fats in the marrows.

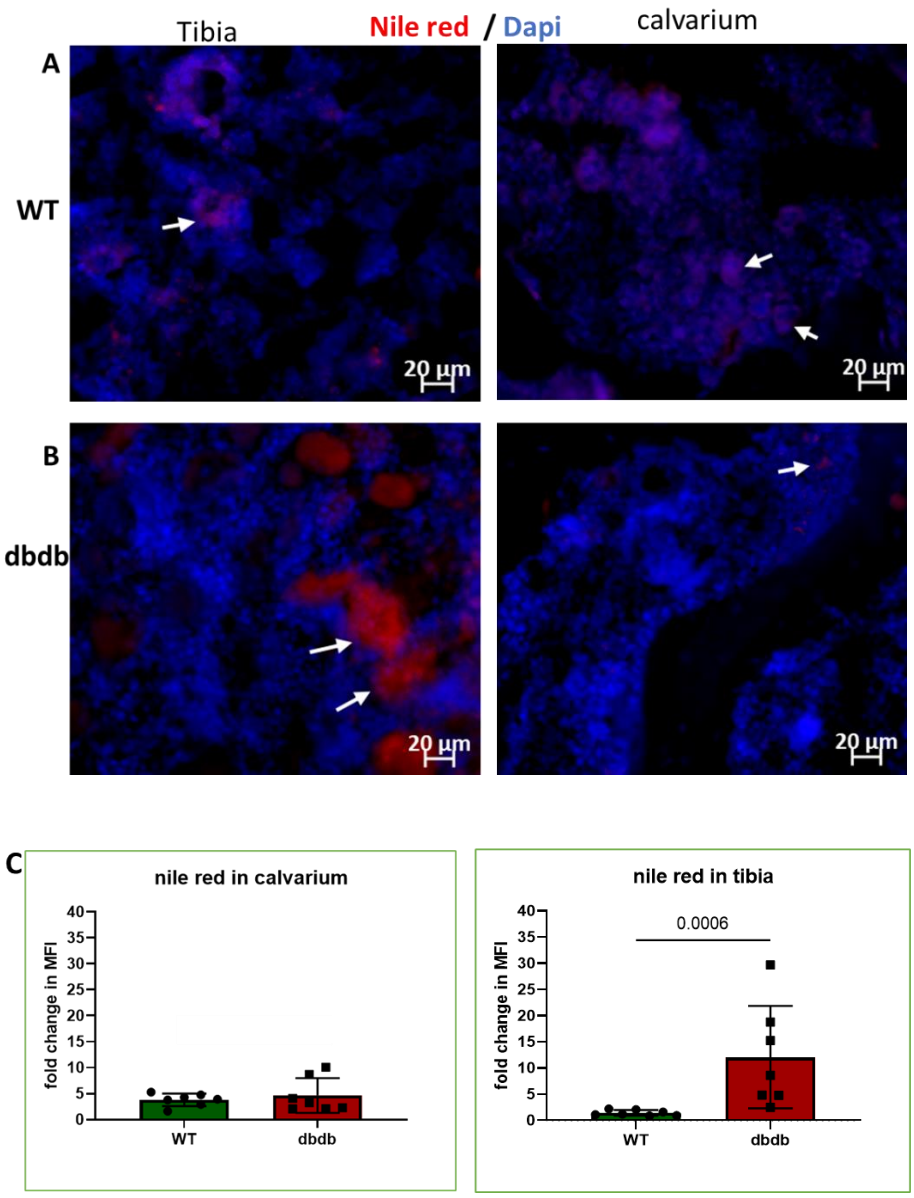


Figure 7. Lipid/Fat contents in the bone marrow. A,B: Representative images showing nile red staining in the calvaria and tibias of control (A) and db/db (B) mice. C: Bar graphs showing quantification of nile red staining, indicating increased fat content in the tibial marrows of db/db mice compared to controls but not in the calvarium.

SUMMARY CONCLUSIONS

Developmental delay in calvarial hematopoiesis and the differential recruitment of bone marrow-derived cells into the injured retina

During embryogenesis, the fetal liver (FL) is seeded with HSCs from the AGM [23, 243]. The FL dominates hematopoiesis prenatally and in the early days of postnatal development. HSCs are seeded into the bone marrow around E16-E18 [244] and shortly after birth, the bone marrow becomes the predominant hematopoietic organ in the adult state. Whereas the bone marrow exists in all long bones (such as tibia and femur) and flat bones (such as the calvarium) in the body [64], development of hematopoiesis in the flat bones has not been investigated as extensively as that of the long bones.

In this project, postnatal hematopoietic development in the calvarium was compared to that of the long bones. The differential contribution of the calvarium and long bones (tibia) to the recruitment of immune cells into the retina following acute ischemic injury was investigated. The primary findings include that the calvarium showed a higher proportion of HSPCs and a lower proportion of mature hematopoietic cells compared to the long bones at P7 and P14 but not at P21. There was a gradual reduction in HSPCs and a concomitant gradual increase in mature hematopoietic cells in the calvarium compared to the long bones over time. These findings suggested a developmental delay in the differentiation potential of calvarial marrow cells. At P14 and

P21 when the bone marrow is known to dominate hematopoiesis, hematopoietic cells of long bone origin reconstituted peripheral blood more rapidly than calvarium cells. Interestingly, however, there was increased engraftment and survival of hematopoietic stem cells and progenitors in the calvarium compared to in the long bones. Upon further investigation, it was observed that the calvarium had increased levels CXCL12, a potent stem cell retention factor [53, 245], compared to the long bones. These results suggested that the calvarial microenvironment provided a better niche promoting more robust engraftment and survival of HSPCs.

This study also investigated the heterogeneity in the mobilization of bone marrow-derived cells into the retina following acute ischemic injury. To test this, bone marrow cells in the calvarium and tibia were labeled by photoconverting the respective bones in KIKGR mice. Using the retinal ischemia-reperfusion injury model, the contribution of each bone marrow site to cells infiltrating the retina post-injury was compared by quantifying the respective proportions of photoconverted cells in the injured retinas. The calvaria marrow contributed more neutrophils and MACs than the long bones (tibia) following acute ischemic retinal injury, even though the proportion of these cells in the marrow at baseline did not differ significantly. This finding pointed to the potential importance of the calvarial marrow to the retina's reparative response to acute injury.

Hematopoietic cells influence vascular development in the retina

It is well established that hematopoietic cells are major players in restoration of retinal health and function following injury of disease. Myeloid angiogenic cells (MACs), neutrophils and monocytes all play unique roles in how the adult retina responses to

inflammation, largely via secretion of paracrine factors that promote cell survival [237]. In addition, it has been shown in animal models that the self-renewal and differentiation ability of hematopoietic stem cells can be utilized to restore the adult retina's health and function in retinal degeneration [246]. Although hematopoiesis begins early in embryogenic development, the role that these cells play in retinal development is underexplored.

The current project characterized the mobilization of myeloid cells into the developing retina and their potential role in the formation of a healthy retina. While there were significant levels of MACs and monocytes, the majority of hematopoietic cells in the developing retina were microglia- cells purported to 'clean' the retina by removing dead and misplaced cells by phagocytosis. We observed hematopoietic cells in close association with the vasculature as the retina's maturation progressed, potentially contributing to the regulation of angiogenesis via paracrine secretions or by targeting retinal astrocytes. Although further investigation is needed to better understand the interaction between hematopoietic cells, astrocytes and endothelial cells in retinal vasculogenesis, this study sheds light on the underestimated role of hematopoietic cells in retinal vasculogenesis and the formation of a healthy retina.

Relative preservation of hematopoiesis in the calvarial marrow in type 2 diabetic mice

The bone marrow suffers diabetic end-organ damage including adipogenesis, microangiopathy and neuropathy [178]. These intra-marrow complications contribute to functional abnormalities such as impaired mobilization of reparative cells and myelopoiesis [202, 221]. However, the impact of diabetes on hematopoiesis at different bone marrow sites has not been studied.

In this study, the effect of long-term type 2 diabetes on the calvarial marrow was compared to that of the tibial marrow. The calvarial and tibial marrows responded differently to type 2 diabetic metabolic disorder. The key findings included that the calvarial marrow showed increased trabecular thickness and bone marrow cellularity. Also, the calvarium preserved the proportion and function of its HSPCs compared to the tibia. In addition, there was less fat accumulation in the calvarial marrow, along with a slower rate of diabetes-associated vascular degeneration. Thus, this study revealed the novel finding that the calvarial marrow undergoes slower diabetes-induced remodeling and improved preservation of hematopoiesis compared to the tibia. The calvarial marrow therefore may serve as a promising source of HSPCs for autologous stem cell therapy in type 2 diabetes. In addition, given its relative ease of access, the calvarial marrow could provide an ideal local target for delivery of ligands that could potentially alleviate diabetes-associated bone marrow pathology.

Limitations

This work has a number of limitations. For cell tracking, we labeled bone marrow-derived cells in vivo by photoconversion- exposing both the calvaria and tibias of KIKGR mice to 405nm laser for 6 minutes each. Differences in bone thickness between the calvarium and tibia potentially affected the penetrance of the laser into the marrows proper. For this reason, not all the bone marrow cells were photoconverted and even though this approach led to a higher percentage of in vivo labeling than what has been reported in the literature for similar experiments [64], the photoconversion rate was different between the two bone marrow locations. Using invasive techniques to further thin the tibia would have damaged the bone and introduced a considerable confounding

factor in the bone marrow's response to retinal injury. Reducing the exposure time of the calvarium to achieve a comparable photoconversion rate to the tibia also would have led to sub-optimal in vivo labelling in the calvarium and affected our ability to track as many calvarial marrow-derived cells as possible. However, this limitation was accounted for by normalizing the experimental data to the baseline rate of each cell type in each distinct location.

In addition, we tracked the recruitment of hematopoietic cells into the developing retina using GFP-labeled Vav1 (Vav1-GFP) transgenic mice. Although it is widely accepted that $Vav1^{+}$ cells are hematopoietic, Vav1 is infrequently activated in endothelial cells [247]. Thus, it is not impossible that a few of the Vav1-GFP cells observed in the developing retina were endothelial in origin. It was beyond the scope of this project to evaluate what proportion of Vav1-GFP cells observed were derived from endothelial cells, if any, and at what point in development this endothelial Vav1 activation occurred. However, the data in this work supports the contribution of hematopoietic cells to retina development given the numerous reports in the literature that Vav1 cells are largely hematopoietic in origin.

Future directions

Hematopoiesis is integral to the maintenance of the circulatory system and the survival of many body systems. It generates the bone marrow cells that are responsible for maintaining the cellular fraction of blood and the repair of tissues after injury. This project has shown that hematopoiesis is different in the distinct bone marrow compartments. Crucially, this work has established that the calvarial marrow microenvironment is specially evolved to promote the engraftment and survival of

hematopoietic stem and progenitor cells in the healthy state and during metabolic stress in type 2 diabetes. While the current work focused on hematopoietic cells in the marrow late in type 2 diabetes, the bone marrow microenvironment comprises a diverse range of cell types including stromal cells of mesenchymal origin and nerve endings [248], all of which experience the metabolic stress caused by diabetes. Future work could investigate the diabetes-associated changes in the composition of bone marrow stromal cells and nerve density in the calvarium versus the tibia by multicolor flow cytometry and immunostaining, respectively. Single cell RNA sequencing will provide further insight into the changes in the epigenetic signature of these bone marrow cells in the calvarium versus tibia in diabetes.

In addition, this work has also shown that the calvarial marrow contributes more myeloid angiogenic cells (MACs) and neutrophils into the retina following acute ischemic injury. Previous studies have shown that MACs become dysfunctional in late diabetes when complications exist [133, 249]. Thus, MACs dysfunction in part explains development of vascular complications and once dysfunctional they become unable to correct vascular damage, contributing to development of diabetic retinopathy. There is still a gap in knowledge regarding the temporal dynamics of recruitment of these cells during chronic conditions such as diabetes. Future work could interrogate which of the two compartments (calvarium versus tibia) contributes more MACs and neutrophils to the retina during early, mid- and late-stage diabetes. In addition, future studies could explore calvarium-targeted delivery of Liver X Receptor (LXR) agonists- cerebrospinal fluid-derived ligands that promote MACs function, to reverse or slow progression of diabetic retinopathy.

GENERAL REFERENCES

1. Smith, C., *Hematopoietic stem cells and hematopoiesis.* Cancer Control, 2003. **10**(1): p. 9-16.

2. Broxmeyer, H.E. and C.H. Kim, *Chemokines and hematopoiesis*, in *Chemokines and cancer*. 1999, Springer. p. 263-291.

3. Palis, J. and M.C. Yoder, *Yolk-sac hematopoiesis: the first blood cells of mouse and man.* Experimental hematology, 2001. **29**(8): p. 927-936.

4. Cumano, A. and I. Godin, *Ontogeny of the hematopoietic system.* Annual review of immunology, 2007. **25**(1): p. 745-785.

5. Kohli, L. and E. Passegué, *Surviving change: the metabolic journey of hematopoietic stem cells.* Trends in cell biology, 2014. **24**(8): p. 479-487.

6. Doulatov, S., et al., *Hematopoiesis: a human perspective.* Cell stem cell, 2012. **10**(2): p. 120-136.

7. Lorenz, E., et al., *Modification of irradiation injury in mice and guinea pigs by bone marrow injections.* Journal of the National Cancer Institute, 1951. **12**(1): p. 197-201.

8. Till, J.E. and E.A. McCulloch, *A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.* Radiation research, 1961. **14**(2): p. 213-222.

9. Moore, M.A. and D. Metcalf, *Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo.* British journal of haematology, 1970. **18**(3): p. 279-296.

10. Orkin, S.H., *Hematopoiesis: how does it happen?* Current opinion in cell biology, 1995. **7**(6): p. 870-877.

11. Scharf, P., et al., *Cellular and molecular mechanisms of environmental pollutants on hematopoiesis.* International Journal of Molecular Sciences, 2020. **21**(19): p. 6996.

12. Verovskaya, E. and G. de Haan, *The power of diversity: hematopoietic stem cell heterogeneity and its clinical relevance.* Clonal analysis of young and aged hematopoietic stem cells using cellular barcoding, 2014. **5**: p. 19.

13. Copelan, E.A., *Hematopoietic stem-cell transplantation.* New England Journal of Medicine, 2006. **354**(17): p. 1813-1826.

14. Sato, T., et al., *Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon–dependent exhaustion.* Nature medicine, 2009. **15**(6): p. 696-700.

15. Essers, M.A., et al., *IFNα activates dormant haematopoietic stem cells in vivo.* Nature, 2009. **458**(7240): p. 904-908.

16. Osawa, M., et al., *Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell.* Science, 1996. **273**(5272): p. 242-245.

17. Mannick, J.A., et al., *Autografts of bone marrow in dogs after lethal total-body radiation.* Blood, 1960. **15**(2): p. 255-266.

18. Thomas, E.D., et al., *Homografts of bone marrow in dogs after lethal total-body radiation.* Blood, 1959. **14**(6): p. 720-736.

19. Gratwohl, A., et al., *Hematopoietic stem cell transplantation: a global perspective.* Jama, 2010. **303**(16): p. 1617-1624.

20. Belyavsky, A., N. Petinati, and N. Drize, *Hematopoiesis during ontogenesis, adult life, and aging.* International Journal of Molecular Sciences, 2021. **22**(17): p. 9231.

21. Medvinsky, A., S. Rybtsov, and S. Taoudi, *Embryonic origin of the adult hematopoietic system: advances and questions.* Development, 2011. **138**(6): p. 1017-1031.

22. Shalaby, F., et al., *A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis.* Cell, 1997. **89**(6): p. 981-990.

23. Yoder, M., *Embryonic hematopoiesis in mice and humans.* Acta Pædiatrica, 2002. **91**: p. 5-8.

24. Ferkowicz, M.J. and M.C. Yoder, *Blood island formation: longstanding observations and modern interpretations.* Experimental hematology, 2005. **33**(9): p. 1041-1047.

25. Palis, J., *Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo.* FEBS letters, 2016. **590**(22): p. 3965-3974.

26. Lin, Y., M.C. Yoder, and M. Yoshimoto, *Lymphoid progenitor emergence in the murine embryo and yolk sac precedes stem cell detection.* Stem cells and development, 2014. **23**(11): p. 1168-1177.

27. Lux, C.T., et al., *All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac.* Blood, The Journal of the American Society of Hematology, 2008. **111**(7): p. 3435-3438.

28. Medvinsky, A. and E. Dzierzak, *Definitive hematopoiesis is autonomously initiated by the AGM region.* Cell, 1996. **86**(6): p. 897-906.

29. Müller, A.M., et al., *Development of hematopoietic stem cell activity in the mouse embryo.* Immunity, 1994. **1**(4): p. 291-301.

30. Medvinsky, A.L., et al., *An early pre-liver intraembryonic source of CFU-S in the developing mouse.* Nature, 1993. **364**(6432): p. 64-67.

31. Taoudi, S., et al., *Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+ CD45+ pre-definitive HSCs.* Cell stem cell, 2008. **3**(1): p. 99-108.

32. Kumaravelu, P., et al., *Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver.* 2002.

33. de Bruijn, M.F., et al., *Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo.* The EMBO journal, 2000. **19**(11): p. 2465-2474.

34. Ciau-Uitz, A., et al., *Developmental hematopoiesis: ontogeny, genetic programming and conservation.* Experimental hematology, 2014. **42**(8): p. 669-683.

35. Dzierzak, E. and A. Medvinsky, *The discovery of a source of adult hematopoietic cells in the embryo.* 2008.

36. Harris, J.M., et al., *Glucose metabolism impacts the spatiotemporal onset and magnitude of HSC induction in vivo.* Blood, The Journal of the American Society of Hematology, 2013. **121**(13): p. 2483-2493.

37. Zovein, A.C., et al., *Fate tracing reveals the endothelial origin of hematopoietic*

stem cells. Cell stem cell, 2008. **3**(6): p. 625-636.

38. Gekas, C., et al., *The placenta is a niche for hematopoietic stem cells.* Developmental cell, 2005. **8**(3): p. 365-375.

39. Alvarez-Silva, M., et al., *Mouse placenta is a major hematopoietic organ.* 2003.
40. Ottersbach, K. and E. Dzierzak, *The murine placenta contains hematopoietic stem cells within the vascular labyrinth region.* Developmental cell, 2005. **8**(3): p. 377-387.

41. Luis, T., N. Killmann, and F. Staal, *Signal transduction pathways regulating hematopoietic stem cell biology: introduction to a series of Spotlight Reviews.* Leukemia, 2012. **26**(1): p. 86-90.

42. Harrison, D., et al., *Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term.* Experimental hematology, 1997. **25**(4): p. 293-297.

43. Rebel, V.I., et al., *The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts.* 1996.

44. Lewis, K., M. Yoshimoto, and T. Takebe, *Fetal liver hematopoiesis: from development to delivery.* Stem Cell Research & Therapy, 2021. **12**(1): p. 1-8.

45. Khan, J.A., et al., *Fetal liver hematopoietic stem cell niches associate with portal vessels.* Science, 2016. **351**(6269): p. 176-180.

46. Yu, H., et al., *Apoptosis and hematopoiesis in murine fetal liver.* 1993.

47. Ema, H. and H. Nakauchi, *Expansion of hematopoietic stem cells in the developing liver of a mouse embryo.* Blood, The Journal of the American Society of Hematology, 2000. **95**(7): p. 2284-2288.

48. Djaldetti, M., H. Bessler, and R.A. Rifkind, *Hematopoiesis in the embryonic mouse spleen: an electron microscopic study.* Blood, 1972. **39**(6): p. 826-841.

49. Bertrand, J.Y., et al., *Fetal spleen stroma drives macrophage commitment.* 2006.

50. Godin, I., et al., *Stem cell emergence and hemopoietic activity are incompatible in mouse intraembryonic sites.* The Journal of experimental medicine, 1999. **190**(1): p. 43-52.

51. Golub, R. and A. Cumano, *Embryonic hematopoiesis.* Blood Cells, Molecules, and Diseases, 2013. **51**(4): p. 226-231.

52. Ciriza, J., et al., *The migration of hematopoietic progenitors from the fetal liver to the fetal bone marrow: lessons learned and possible clinical applications.* Experimental hematology, 2013. **41**(5): p. 411-423.

53. Christensen, J.L., et al., *Circulation and chemotaxis of fetal hematopoietic stem cells.* PLoS biology, 2004. **2**(3): p. e75.

54. Hall, T.D., et al., *Murine fetal bone marrow does not support functional hematopoietic stem and progenitor cells until birth.* Nature Communications, 2022. **13**(1): p. 1-18.

55. Coskun, S., et al., *Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. Cell Rep. 2014; 9 (2): 581-90*. Epub 2014/10/15. doi: 10.1016/j. celrep. 2014.09. 013 S2211-1247 (14) 00781 ….

56. Morrison, S.J., et al., *The purification and characterization of fetal liver hematopoietic stem cells.* Proceedings of the National Academy of Sciences, 1995. **92**(22): p. 10302-10306.

57. Ahmadbeigi, N., et al., *Isolation, characterization, and transplantation of bone marrow-derived cell components with hematopoietic stem cell niche properties.* Stem cells and development, 2013. **22**(23): p. 3052-3061.

58. Mendelson, A. and P.S. Frenette, *Hematopoietic stem cell niche maintenance during homeostasis and regeneration.* Nature medicine, 2014. **20**(8): p. 833-846.

59. Heazlewood, S.Y., et al., *Analyzing hematopoietic stem cell homing, lodgment, and engraftment to better understand the bone marrow niche.* Annals of the New York Academy of Sciences, 2014. **1310**(1): p. 119-128.

60. Hajishengallis, G., X. Li, and T. Chavakis, *Immunometabolic control of hematopoiesis.* Molecular Aspects of Medicine, 2021. **77**: p. 100923.

61. Nakamura-Ishizu, A., K. Ito, and T. Suda, *Hematopoietic stem cell metabolism during development and aging.* Developmental cell, 2020. **54**(2): p. 239-255.

62. Ito, K. and T. Suda, *Metabolic requirements for the maintenance of self-renewing stem cells.* Nature reviews Molecular cell biology, 2014. **15**(4): p. 243-256.

63. Kiel, M.J. and S.J. Morrison, *Uncertainty in the niches that maintain haematopoietic stem cells.* Nature Reviews Immunology, 2008. **8**(4): p. 290-301.

64. Herisson, F., et al., *Direct vascular channels connect skull bone marrow and the brain surface enabling myeloid cell migration.* Nature neuroscience, 2018. **21**(9): p. 1209-1217.

65. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology.* Cell, 2008. **132**(4): p. 631-644.

66. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.* cell, 2005. **121**(7): p. 1109-1121.

67. Morrison, S.J. and D.T. Scadden, *The bone marrow niche for haematopoietic stem cells.* Nature, 2014. **505**(7483): p. 327-334.

68. Wilson, A. and A. Trumpp, *Bone-marrow haematopoietic-stem-cell niches.* Nature Reviews Immunology, 2006. **6**(2): p. 93-106.

69. Chen, F., Y. Han, and Y. Kang, *Bone marrow niches in the regulation of bone metastasis.* British Journal of Cancer, 2021. **124**(12): p. 1912-1920.

70. Crane, G.M., E. Jeffery, and S.J. Morrison, *Adult haematopoietic stem cell niches.* Nature Reviews Immunology, 2017. **17**(9): p. 573-590.

71. Lymperi, S., et al., *Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo.* Blood, The Journal of the American Society of Hematology, 2011. **117**(5): p. 1540-1549.

72. Visnjic, D., et al., *Hematopoiesis is severely altered in mice with an induced osteoblast deficiency.* Blood, 2004. **103**(9): p. 3258-3264.

73. Calvi, L., et al., *Osteoblastic cells regulate the haematopoietic stem cell niche.* Nature, 2003. **425**(6960): p. 841-846.

74. Kiel, M.J., G.L. Radice, and S.J. Morrison, *Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance.* Cell stem cell, 2007. **1**(2): p. 204-217.

75. Méndez-Ferrer, S., et al., *Mesenchymal and haematopoietic stem cells form a unique bone marrow niche.* nature, 2010. **466**(7308): p. 829-834.

76. Omatsu, Y., et al., *The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche.* Immunity, 2010. **33**(3): p. 387-399.

77. Psaila, B., D. Lyden, and I. Roberts, *Megakaryocytes, malignancy and bone marrow vascular niches.* Journal of Thrombosis and Haemostasis, 2012. **10**(2): p. 177-188.

78. Morrison, S.J. and A.C. Spradling, *Stem cells and niches: mechanisms that promote stem cell maintenance throughout life.* Cell, 2008. **132**(4): p. 598-611.

79. Gimble, J., et al., *The function of adipocytes in the bone marrow stroma: an update.* Bone, 1996. **19**(5): p. 421-428.

80. Kopp, H.-G., et al., *The bone marrow vascular niche: home of HSC differentiation and mobilization.* Physiology, 2005. **20**(5): p. 349-356.

81. Fliedner, T.M., et al., *Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure.* Cancer Biotherapy and Radiopharmaceuticals, 2002. **17**(4): p. 405-426.

82. Kiel, M.J. and S.J. Morrison, *Maintaining hematopoietic stem cells in the vascular niche.* Immunity, 2006. **25**(6): p. 862-864.

83. Garrett, R.W. and S.G. Emerson, *Bone and blood vessels: the hard and the soft of hematopoietic stem cell niches.* Cell stem cell, 2009. **4**(6): p. 503-506.

84. Ding, L., et al., *Endothelial and perivascular cells maintain haematopoietic stem cells.* Nature, 2012. **481**(7382): p. 457-462.

85. Mansour, A., et al., *Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow.* Journal of Experimental Medicine, 2012. **209**(3): p. 537-549.

86. Adams, G.B., et al., *Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor.* Nature, 2006. **439**(7076): p. 599-603.

87. Petit, I., et al., *G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4.* Nature immunology, 2002. **3**(7): p. 687-694.

88. Yoshihara, H., et al., *Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche.* Cell stem cell, 2007. **1**(6): p. 685-697.

89. Arai, F., et al., *Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche.* Cell, 2004. **118**(2): p. 149-161.

90. Sugiyama, T., et al., *Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches.* Immunity, 2006. **25**(6): p. 977-988.

91. Itkin, T. and T. Lapidot, *SDF-1 keeps HSC quiescent at home.* Blood, The Journal of the American Society of Hematology, 2011. **117**(2): p. 373-374.

92. Nie, Y., Y.-C. Han, and Y.-R. Zou, *CXCR4 is required for the quiescence of primitive hematopoietic cells.* The Journal of experimental medicine, 2008. **205**(4): p. 777-783.

93. Yamazaki, K. and T.D. Allen, *Ultrastructural morphometric study of efferent nerve terminals on murine bone marrow stromal cells, and the recognition of a novel anatomical unit: The "neuro-reticular complex".* American Journal of Anatomy, 1990. **187**(3): p. 261-276.

94. Tabarowski, Z., K. Gibson-Berry, and S.Y. Felten, *Noradrenergic and peptidergic innervation of the mouse femur bone marrow.* Acta histochemica, 1996. **98**(4): p. 453-457.

95. Bellinger, D.L., et al., *Innervation of lymphoid organs and implications in development, aging, and autoimmunity.* International journal of immunopharmacology, 1992. **14**(3): p. 329-344.

96. Marino, F., et al., *Measurement of catecholamines in mouse bone marrow by means of HPLC with electrochemical detection.* Haematologica, 1997. **82**(4): p. 392-394.

97. Bjurholm, A., *Neuroendocrine peptides in bone.* International orthopaedics, 1991. **15**(4): p. 325-329.

98. Bjurholm, A., et al., *The occurrence of neuropeptides at different stages of DBM-induced heterotopic bone formation.* Bone and mineral, 1990. **10**(2): p. 95-107.

99. Fadini, G.P., et al., *Concise review: diabetes, the bone marrow niche, and impaired vascular regeneration.* Stem cells translational medicine, 2014. **3**(8): p. 949-957.

100. Spiegel, A., et al., *Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling.* Nature immunology, 2007. **8**(10): p. 1123-1131.

101. Lucas, D., et al., *Mobilized hematopoietic stem cell yield depends on species-specific circadian timing.* Cell stem cell, 2008. **3**(4): p. 364-366.

102. Dar, A., et al., *Rapid mobilization of hematopoietic progenitors by AMD3100 and catecholamines is mediated by CXCR4-dependent SDF-1 release from bone marrow stromal cells.* Leukemia, 2011. **25**(8): p. 1286-1296.

103. Suekane, A., et al., *CGRP-CRLR/RAMP1 signal is important for stress-induced hematopoiesis.* Scientific reports, 2019. **9**(1): p. 1-11.

104. Mullins, M.W., et al., *Characterization of a calcitonin gene-related peptide (CGRP) receptor on mouse bone marrow cells.* Regulatory peptides, 1993. **49**(1): p. 65-72.

105. Kosaras, B., et al., *Sensory innervation of the calvarial bones of the mouse.*

Journal of Comparative Neurology, 2009. **515**(3): p. 331-348.

106. Gao, X., et al., *Nociceptive nerves regulate haematopoietic stem cell mobilization.* Nature, 2021. **589**(7843): p. 591-596.

107. Lam, B.S., C. Cunningham, and G.B. Adams, *Pharmacologic modulation of the calcium-sensing receptor enhances hematopoietic stem cell lodgment in the adult bone marrow.* Blood, The Journal of the American Society of Hematology, 2011. **117**(4): p. 1167-1175.

108. Jiang, X., et al., *Tissue origins and interactions in the mammalian skull vault.* Developmental biology, 2002. **241**(1): p. 106-116.

109. Ishii, M., et al., *The development of the calvarial bones and sutures and the pathophysiology of craniosynostosis.* Current topics in developmental biology, 2015. **115**: p. 131-156.

110. Marghoub, A., et al., *Characterizing and modeling bone formation during mouse calvarial development.* Physical review letters, 2019. **122**(4): p. 048103.

111. Adams, G.B. and D.T. Scadden, *The hematopoietic stem cell in its place.* Nature immunology, 2006. **7**(4): p. 333-337.

112. Tarnowski, C.P., M.A. Ignelzi Jr, and M.D. Morris, *Mineralization of developing mouse calvaria as revealed by Raman microspectroscopy.* Journal of Bone and Mineral Research, 2002. **17**(6): p. 1118-1126.

113. Nakano, T., et al., *Development of erythroid cells from mouse embryonic stem cells in culture: potential use for erythroid transcription factor study.* Leukemia (08876924), 1997. **11**.

114. Kitajima, K., et al., *In vitro differentiation of mouse embryonic stem cells to hematopoietic cells on an OP9 stromal cell monolayer.* Methods in enzymology, 2003. **365**: p. 72-83.

115. Nakano, T., H. Kodama, and T. Honjo, *Generation of lymphohematopoietic cells from embryonic stem cells in culture.* Science, 1994. **265**(5175): p. 1098-1101.

116. Lo Celso, C., C.P. Lin, and D.T. Scadden, *In vivo imaging of transplanted hematopoietic stem and progenitor cells in mouse calvarium bone marrow.* Nature protocols, 2011. **6**(1): p. 1-14.

117. Kim, S., et al., *Extended time-lapse in vivo imaging of tibia bone marrow to visualize dynamic hematopoietic stem cell engraftment.* Leukemia, 2017. **31**(7): p. 1582-1592.

118. Sipkins, D.A., et al., *In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment.* Nature, 2005. **435**(7044): p. 969-973.

119. Kopp, H.G., et al., *Functional heterogeneity of the bone marrow vascular niche.* Annals of the New York Academy of Sciences, 2009. **1176**(1): p. 47-54.

120. Nombela-Arrieta, C., et al., *Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment.* Nature cell biology, 2013. **15**(5): p. 533-543.

121. Li, X.-M., et al., *High levels of acetylated low-density lipoprotein uptake and low tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) promoter activity distinguish sinusoids from other vessel types in murine bone marrow.* Circulation, 2009. **120**(19): p. 1910-1918.

122. Mazo, I.B., et al., *Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1.* The Journal of experimental medicine, 1998. **188**(3): p. 465-474.

123. Poller, W.C., et al., *Brain motor and fear circuits regulate leukocytes during acute stress.* Nature, 2022: p. 1-3.

124. Pulous, F.E., et al., *Cerebrospinal fluid can exit into the skull bone marrow and instruct cranial hematopoiesis in mice with bacterial meningitis.* Nature Neuroscience, 2022. **25**(5): p. 567-576.

125. Greene, R. and D. Kochhar, *Limb development in mouse embryos: protection against teratogenic effects of 6-diazo-5 oxo-L-norleucine (DON) in vivo and in vitro.* 1975.

126. Hankenson, K.D., et al., *Mice with a disruption of the thrombospondin 3 gene differ in geometric and biomechanical properties of bone and have accelerated development of the femoral head.* Molecular and cellular biology, 2005. **25**(13): p. 5599-5606.

127. Martin, G.R., *The roles of FGFs in the early development of vertebrate limbs.* Genes & development, 1998. **12**(11): p. 1571-1586.

128. Oftadeh, R., et al., *Biomechanics and mechanobiology of trabecular bone: a review.* Journal of biomechanical engineering, 2015. **137**(1).

129. Rüegsegger, P., E. Durand, and M. Dambacher, *Differential effects of aging and disease on trabecular and compact bone density of the radius.* Bone, 1991. **12**(2): p. 99-105.

130. Zhang, J., et al., *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature, 2003. **425**(6960): p. 836-841.

131. Gong, J.K., *Endosteal marrow: a rich source of hematopoietic stem cells.* Science, 1978. **199**(4336): p. 1443-1445.

132. Guezguez, B., et al., *Regional localization within the bone marrow influences the functional capacity of human HSCs.* Cell stem cell, 2013. **13**(2): p. 175-189.

133. Caballero, S., et al., *Circulating mononuclear progenitor cells: differential roles for subpopulations in repair of retinal vascular injury.* Investigative ophthalmology & visual science, 2013. **54**(4): p. 3000-3009.

134. Crosby, J.R., et al., *Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation.* Circulation research, 2000. **87**(9): p. 728-730.

135. Tchernychev, B., et al., *Discovery of a CXCR4 agonist pepducin that mobilizes bone marrow hematopoietic cells.* Proceedings of the National Academy of Sciences, 2010. **107**(51): p. 22255-22259.

136. Evrard, M., et al., *Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions.* Immunity, 2018. **48**(2): p. 364-379. e8.

137. Ludin, A., et al., *Monocytes-macrophages that express α-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow.* Nature immunology, 2012. **13**(11): p. 1072-1082.

138. Tokoyoda, K., et al., *Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow.* Immunity, 2009. **30**(5): p. 721-730.

139. Rafii, S. and D. Lyden, *Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration.* Nature medicine, 2003. **9**(6): p. 702-712.

140. Otani, A., et al., *Bone marrow–derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis.* Nature medicine, 2002. **8**(9): p. 1004-1010.

141. Sata, M., et al., *Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis.* Nature medicine, 2002. **8**(4): p. 403-409.

142. Edelberg, J.M., et al., *Young adult bone marrow–derived endothelial precursor cells restore aging-impaired cardiac angiogenic function.* Circulation research, 2002. **90**(10): p. e89-e93.

143. Caballero, S., et al., *Bone Marrow–Derived Cell Recruitment to the Neurosensory Retina and Retinal Pigment Epithelial Cell Layer Following Subthreshold Retinal Phototherapy.* Investigative ophthalmology & visual science, 2017. **58**(12): p. 5164-5176.

144. Chakravarthy, H., et al., *Imbalances in mobilization and activation of pro-inflammatory and vascular reparative bone marrow-derived cells in diabetic retinopathy.* PloS one, 2016. **11**(1): p. e0146829.

145. Sasahara, M., et al., *Activation of bone marrow-derived microglia promotes photoreceptor survival in inherited retinal degeneration.* The American journal of pathology, 2008. **172**(6): p. 1693-1703.

146. Purcell, B.P., et al., *Synergistic effects of SDF-1α chemokine and hyaluronic acid release from degradable hydrogels on directing bone marrow derived cell homing to the myocardium.* Biomaterials, 2012. **33**(31): p. 7849-7857.

147. Kielar, M.L., et al., *Maladaptive role of IL-6 in ischemic acute renal failure.* Journal of the American Society of Nephrology, 2005. **16**(11): p. 3315-3325.

148. Tsung, A., et al., *Hepatic ischemia/reperfusion injury involves functional TLR4 signaling in nonparenchymal cells.* The Journal of Immunology, 2005. **175**(11): p. 7661-7668.

149. Mollen, K.P., et al., *Systemic inflammation and end organ damage following trauma involves functional TLR4 signaling in both bone marrow-derived cells and parenchymal cells.* Journal of leukocyte biology, 2008. **83**(1): p. 80-88.

150. Heissig, B., et al., *Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand.* Cell, 2002. **109**(5): p. 625-637.

151. Hattori, K., et al., *Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1+ stem cells from bone-marrow microenvironment.* Nature medicine, 2002. **8**(8): p. 841-849.

152. Quarmby, S., P. Kumar, and S. Kumar, *Radiation-induced normal tissue injury: Role of adhesion molecules in leukocyte–endothelial cell interactions.* International Journal of Cancer, 1999. **82**(3): p. 385-395.

153. Lawrence, M.B. and T.A. Springer, *Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins.* Cell, 1991. **65**(5): p. 859-873.

154. Gumina, R.J., et al., *Antibody to platelet/endothelial cell adhesion molecule-1 reduces myocardial infarct size in a rat model of ischemia-reperfusion injury.*

Circulation, 1996. **94**(12): p. 3327-3333.

155. Kurose, I., et al., *Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage.* Circulation Research, 1994. **74**(2): p. 336-343.

156. Kim, S.Y., et al., *Neutrophils are associated with capillary closure in spontaneously diabetic monkey retinas.* Diabetes, 2005. **54**(5): p. 1534-1542.

157. Liu, Y., et al., *Reversible retinal vessel closure from VEGF-induced leukocyte plugging.* JCI insight, 2017. **2**(18).

158. Joussen, A.M., et al., *Leukocyte-mediated endothelial cell injury and death in the diabetic retina.* The American journal of pathology, 2001. **158**(1): p. 147-152.

159. Ghosh, S., et al., *Neutrophils homing into the retina trigger pathology in early age-related macular degeneration.* Communications biology, 2019. **2**(1): p. 1-17.

160. Gnecchi, M., et al., *Paracrine mechanisms in adult stem cell signaling and therapy.* Circulation research, 2008. **103**(11): p. 1204-1219.

161. London, A., et al., *Neuroprotection and progenitor cell renewal in the injured adult murine retina requires healing monocyte-derived macrophages.* Journal of Experimental Medicine, 2011. **208**(1): p. 23-39.

162. Fletcher, E.L., *Contribution of microglia and monocytes to the development and progression of age related macular degeneration.* Ophthalmic and Physiological Optics, 2020. **40**(2): p. 128-139.

163. Kurimoto, T., et al., *Neutrophils express oncomodulin and promote optic nerve regeneration.* Journal of Neuroscience, 2013. **33**(37): p. 14816-14824.

164. Chan-Ling, T., et al., *Hematopoietic stem cells provide repair functions after laser-induced Bruch's membrane rupture model of choroidal neovascularization.* The American journal of pathology, 2006. **168**(3): p. 1031-1044.

165. Asare-Bediako, B., et al., *Hematopoietic Cells Influence Vascular Development in the Retina.* Cells, 2022. **11**(20): p. 3207.

166. Grant, M.B., et al., *Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization.* Nature medicine, 2002. **8**(6): p. 607-612.

167. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* New England journal of medicine, 2005. **352**(16): p. 1685-1695.

168. Kamari, Y., et al., *Reduced atherosclerosis and inflammatory cytokines in apolipoprotein-E-deficient mice lacking bone marrow-derived interleukin-1α.* Biochemical and biophysical research communications, 2011. **405**(2): p. 197-203.

169. Kishimoto, T., et al., *Interleukin-6 family of cytokines and gp130.* 1995.

170. Ikebuchi, K., et al., *Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors.* Proceedings of the National Academy of Sciences, 1987. **84**(24): p. 9035-9039.

171. Dobbs, R., et al., *Glucagon: role in the hyperglycemia of diabetes mellitus.* Science, 1975. **187**(4176): p. 544-547.

172. Magaji, V. and J.M. Johnston, *Inpatient management of hyperglycemia and diabetes.* Clinical Diabetes, 2011. **29**(1): p. 3-9.

173. Ceriello, A., *Postprandial hyperglycemia and diabetes complications: is it time to treat?* Diabetes, 2005. **54**(1): p. 1-7.

174. Stern, M.P. and S.M. Haffner, *Body fat distribution and hyperinsulinemia as risk factors for diabetes and cardiovascular disease.* Arteriosclerosis: An Official Journal of the American Heart Association, Inc., 1986. **6**(2): p. 123-130.

175. Kulkarni, R.N., et al., *Impact of genetic background on development of hyperinsulinemia and diabetes in insulin receptor/insulin receptor substrate-1 double heterozygous mice.* Diabetes, 2003. **52**(6): p. 1528-1534.

176. Solano, M.P. and R.B. Goldberg, *Management of dyslipidemia in diabetes.* Cardiology in review, 2006. **14**(3): p. 125-135.

177. Mooradian, A.D., *Dyslipidemia in type 2 diabetes mellitus.* Nature Reviews Endocrinology, 2009. **5**(3): p. 150-159.

178. Fadini, G.P., S. Ciciliot, and M. Albiero, *Concise review: perspectives and clinical implications of bone marrow and circulating stem cell defects in diabetes.* Stem Cells, 2017. **35**(1): p. 106-116.

179. Oikawa, A., et al., *Diabetes mellitus induces bone marrow microangiopathy.* Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(3): p. 498-508.

180. Santopaolo, M., et al., *Bone marrow as a target and accomplice of vascular complications in diabetes.* Diabetes/Metabolism Research and Reviews, 2020. **36**: p. e3240.

181. Keats, E.C. and Z.A. Khan, *Vascular stem cells in diabetic complications: evidence for a role in the pathogenesis and the therapeutic promise.*

Cardiovascular diabetology, 2012. **11**(1): p. 1-10.

182. Khan, Z.A. and S. Chakrabarti, *Therapeutic targeting of endothelial dysfunction in chronic diabetic complications.* Recent Patents on Cardiovascular Drug Discovery (Discontinued), 2006. **1**(2): p. 167-175.

183. Boeri, D., et al., *Modification of tissue-factor mRNA and protein response to thrombin and interleukin 1 by high glucose in cultured human endothelial cells.* Diabetes, 1989. **38**(2): p. 212-218.

184. Graier, W.F., et al., *Intracellular mechanism of highd-glucose-induced modulation of vascular cell proliferation.* European journal of pharmacology, 1995. **294**(1): p. 221-229.

185. Mangialardi, G., et al., *Diabetes causes bone marrow endothelial barrier dysfunction by activation of the RhoA–Rho-associated kinase signaling pathway.* Arteriosclerosis, thrombosis, and vascular biology, 2013. **33**(3): p. 555-564.

186. Albiero, M., et al., *Diabetes causes bone marrow autonomic neuropathy and impairs stem cell mobilization via dysregulated p66Shc and Sirt1.* Diabetes, 2014. **63**(4): p. 1353-1365.

187. Busik, J.V., et al., *Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock.* Journal of Experimental Medicine, 2009. **206**(13): p. 2897-2906.

188. Ferraro, F., et al., *Diabetes impairs hematopoietic stem cell mobilization by altering niche function.* Science translational medicine, 2011. **3**(104): p. 104ra101-104ra101.

189. Orlandi, A., et al., *Long-term diabetes impairs repopulation of hematopoietic progenitor cells and dysregulates the cytokine expression in the bone marrow microenvironment in mice.* Basic research in cardiology, 2010. **105**(6): p. 703-712.

190. Fadini, G.P., et al., *Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy.* Arteriosclerosis, thrombosis, and vascular biology, 2006. **26**(9): p. 2140-2146.

191. Tepper, O.M., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-2786.

192. Fadini, G.P., et al., *Time course and mechanisms of circulating progenitor cell reduction in the natural history of type 2 diabetes.* Diabetes care, 2010. **33**(5): p. 1097-1102.

193. Fadini, G.P., et al., *Stem cell compartmentalization in diabetes and high cardiovascular risk reveals the role of DPP-4 in diabetic stem cell mobilopathy.* Basic research in cardiology, 2013. **108**(1): p. 1-12.

194. Loomans, C.J., et al., *Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes.* Diabetes, 2004. **53**(1): p. 195-199.

195. Katayama, Y., et al., *Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow.* Cell, 2006. **124**(2): p. 407-421.

196. Méndez-Ferrer, S., et al., *Haematopoietic stem cell release is regulated by circadian oscillations.* Nature, 2008. **452**(7186): p. 442-447.

197. Woo, S.J., et al., *Elevated systemic neutrophil count in diabetic retinopathy and diabetes: a hospital-based cross-sectional study of 30,793 Korean subjects.* Investigative ophthalmology & visual science, 2011. **52**(10): p. 7697-7703.

198. Barrett, T.J., et al., *Diabetes-mediated myelopoiesis and the relationship to cardiovascular risk.* Annals of the New York Academy of Sciences, 2017. **1402**(1): p. 31-42.

199. Ford, E.S., *Leukocyte count, erythrocyte sedimentation rate, and diabetes incidence in a national sample of US adults.* American journal of epidemiology, 2002. **155**(1): p. 57-64.

200. Ohshita, K., et al., *Elevated white blood cell count in subjects with impaired glucose tolerance.* Diabetes care, 2004. **27**(2): p. 491-496.

201. Kullo, I.J., D.D. Hensrud, and T.G. Allison, *Comparison of numbers of circulating blood monocytes in men grouped by body mass index (< 25, 25 to< 30,≥ 30).* American Journal of Cardiology, 2002. **89**(12): p. 1441-1443.

202. Albiero, M., et al., *Diabetes-associated myelopoiesis drives stem cell mobilopathy through an OSM-p66Shc signaling pathway.* Diabetes, 2019. **68**(6): p. 1303-1314.

203. Hazra, S., et al., *Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model.* Diabetologia, 2013. **56**(3): p. 644-653.

204. Nagareddy, P.R., et al., *Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis.* Cell metabolism, 2013. **17**(5): p. 695-708.

205. Hoyer, F.F., et al., *Bone marrow endothelial cells regulate myelopoiesis in diabetes mellitus.* Circulation, 2020. **142**(3): p. 244-258.

206. Preusch, M.R., et al., *Deletion of bone marrow derived receptor for advanced glycation end-products (RAGE) does not attenuate plaque formation but inhibits plaque progression in a mouse model of advanced atherosclerosis*. 2009, Am Heart Assoc.

207. Soro-Paavonen, A., et al., *Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes.* Diabetes, 2008. **57**(9): p. 2461-2469.

208. Nagareddy, P.R., et al., *Adipose tissue macrophages promote myelopoiesis and monocytosis in obesity.* Cell metabolism, 2014. **19**(5): p. 821-835.

209. Luo, Y., et al., *Microbiota from obese mice regulate hematopoietic stem cell differentiation by altering the bone niche.* Cell metabolism, 2015. **22**(5): p. 886-894.

210. Spinetti, G., et al., *Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microRNA-155/FOXO3a signaling pathway.* Circulation research, 2013. **112**(3): p. 510-522.

211. Naveiras, O., et al., *Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment.* Nature, 2009. **460**(7252): p. 259-263.

212. Keats, E. and Z.A. Khan, *Unique responses of stem cell-derived vascular endothelial and mesenchymal cells to high levels of glucose.* PloS one, 2012. **7**(6): p. e38752.

213. Botolin, S. and L.R. McCabe, *Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice.* Endocrinology, 2007. **148**(1): p. 198-205.

214. Keats, E.C., et al., *Switch from canonical to noncanonical Wnt signaling mediates high glucose-induced adipogenesis.* Stem Cells, 2014. **32**(6): p. 1649-1660.

215. Kim, T.Y. and A.L. Schafer, *Diabetes and bone marrow adiposity.* Current osteoporosis reports, 2016. **14**(6): p. 337-344.

216. Botolin, S., et al., *Increased bone adiposity and peroxisomal proliferator-activated receptor-γ2 expression in type I diabetic mice.* Endocrinology, 2005. **146**(8): p. 3622-3631.

217. Piccinin, M.A. and Z.A. Khan, *Pathophysiological role of enhanced bone marrow adipogenesis in diabetic complications.* Adipocyte, 2014. **3**(4): p. 263-272.

218. Camp, H.S., D. Ren, and T. Leff, *Adipogenesis and fat-cell function in obesity and diabetes.* Trends in molecular medicine, 2002. **8**(9): p. 442-447.

219. Lecka-Czernik, B., et al., *Divergent effects of selective peroxisome proliferator-activated receptor-γ2 ligands on adipocyte versus osteoblast differentiation.* Endocrinology, 2002. **143**(6): p. 2376-2384.

220. Kojima, H., J. Kim, and L. Chan, *Emerging roles of hematopoietic cells in the pathobiology of diabetic complications.* Trends in Endocrinology & Metabolism, 2014. **25**(4): p. 178-187.

221. Fadini, G.P., et al., *Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus.* Journal of the American College of Cardiology, 2005. **45**(9): p. 1449-1457.

222. Vieira, C.P., et al., *Selective LXR agonist DMHCA corrects retinal and bone marrow dysfunction in type 2 diabetes.* JCI insight, 2020. **5**(13).

223. Duan, Y., et al., *Loss of angiotensin-converting enzyme 2 exacerbates diabetic retinopathy by promoting bone marrow dysfunction.* Stem Cells, 2018. **36**(9): p. 1430-1440.

224. Chartier, S.R., et al., *The changing sensory and sympathetic innervation of the young, adult and aging mouse femur.* Neuroscience, 2018. **387**: p. 178-190.

225. Isidro, R.A., et al., *Double immunofluorescent staining of rat macrophages in formalin-fixed paraffin-embedded tissue using two monoclonal mouse antibodies.* Histochemistry and cell biology, 2015. **144**(6): p. 613-621.

226. Lin, Y., et al., *Activation of osteoblast ferroptosis via the METTL3/ASK1-p38 signaling pathway in high glucose and high fat (HGHF)-induced diabetic bone loss.* The FASEB Journal, 2022. **36**(3): p. e22147.

227. Mohsin, S., et al., *An update on therapies for the treatment of diabetes-induced osteoporosis.* Expert Opinion on Biological Therapy, 2019. **19**(9): p. 937-948.

228. Hamann, C., et al., *Bone, sweet bone—osteoporotic fractures in diabetes mellitus.* Nature Reviews Endocrinology, 2012. **8**(5): p. 297-305.

229. Catalfamo, D.L., et al., *Hyperglycemia induced and intrinsic alterations in type 2 diabetes-derived osteoclast function.* Oral diseases, 2013. **19**(3): p. 303-312.

230. Yang, M., et al., *Tubeimoside I suppresses diabetes-induced bone loss in rats, osteoclast formation, and RANKL-induced nuclear factor-κB pathway.* International Immunopharmacology, 2020. **80**: p. 106202.

231. Pritchard, J.M., et al., *Association of larger holes in the trabecular bone at the distal radius in postmenopausal women with type 2 diabetes mellitus compared to controls.* Arthritis care & research, 2012. **64**(1): p. 83-91.

232. Rathinavelu, S., C. Guidry-Elizondo, and J. Banu, *Molecular modulation of osteoblasts and osteoclasts in type 2 diabetes.* Journal of diabetes research, 2018. **2018**.

233. Schwartz, A.V. and D.E. Sellmeyer, *Women, type 2 diabetes, and fracture risk.* Current diabetes reports, 2004. **4**(5): p. 364-369.

234. Ferland-McCollough, D., et al., *MCP-1 feedback loop between adipocytes and mesenchymal stromal cells causes fat accumulation and contributes to hematopoietic stem cell rarefaction in the bone marrow of patients with diabetes.* Diabetes, 2018. **67**(7): p. 1380-1394.

235. Greenspan, P., E.P. Mayer, and S.D. Fowler, *Nile red: a selective fluorescent stain for intracellular lipid droplets.* The Journal of cell biology, 1985. **100**(3): p. 965-973.

236. Egan, C., et al., *Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes.* Diabetologia, 2008. **51**(7): p. 1296-1305.

237. Bhatwadekar, A.D., et al., *Hematopoietic stem/progenitor involvement in retinal microvascular repair during diabetes: Implications for bone marrow rejuvenation.* Vision research, 2017. **139**: p. 211-220.

238. Chakravarthy, H., et al., *Role of acid sphingomyelinase in shifting the balance between proinflammatory and reparative bone marrow cells in diabetic retinopathy.* Stem cells, 2016. **34**(4): p. 972-983.

239. Theofilopoulos, S. and E. Arenas, *Liver X receptors and cholesterol metabolism: role in ventral midbrain development and neurodegeneration.* F1000prime Reports, 2015. **7**.

240. Mills III, W.A., M.A. Coburn, and U.B. Eyo, *The emergence of the calvarial hematopoietic niche in health and disease.* Immunological Reviews, 2022.

241. Bjurstrom, M.F., S.E. Giron, and C.A. Griffis, *Cerebrospinal fluid cytokines and neurotrophic factors in human chronic pain populations: a comprehensive review.* Pain Practice, 2016. **16**(2): p. 183-203.

242. Eventov, I., et al., *Osteopenia, hematopoiesis, and bone remodelling in iliac crest and femoral biopsies: a prospective study of 102 cases of femoral neck fractures.* Bone, 1991. **12**(1): p. 1-6.

243. Rieger, M.A. and T. Schroeder, *Hematopoiesis.* Cold Spring Harbor perspectives in biology, 2012. **4**(12): p. a008250.

244. Galloway, J.L. and L.I. Zon, *3 ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo.* 2003.

245. Suárez-Álvarez, B., A. López-Vázquez, and C. López-Larrea, *Mobilization and homing of hematopoietic stem cells.* Stem cell transplantation, 2012: p. 152-170.

246. Sengupta, N., et al., *Regulation of adult hematopoietic stem cells fate for enhanced tissue-specific repair.* Molecular Therapy, 2009. **17**(9): p. 1594-1604.

247. Hong, J., et al., *Vav1 is Essential for HIF-1α Activation via a Lysosomal VEGFR1-Mediated Degradation Mechanism in Endothelial Cells.* Cancers, 2020. **12**(6): p. 1374.

248. Tikhonova, A.N., et al., *The bone marrow microenvironment at single-cell resolution.* Nature, 2019. **569**(7755): p. 222-228.

249. Jarajapu, Y.P., et al., *Vasoreparative dysfunction of CD34+ cells in diabetic individuals involves hypoxic desensitization and impaired autocrine/paracrine mechanisms.* PloS one, 2014. **9**(4): p. e93965.

APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 13-Jun-2018

TO: Grant, Maria Bartolomeo

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 13-Jun-2018.

Protocol PI:	Grant, Maria Bartolomeo
Title:	Effect of a Novel anti-VEGF therapy on a mouse model of AMD.
Sponsor:	UAB DEPARTMENT
Animal Project Number (APN):	IACUC-21261

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 12-Jun-2021.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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