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Benjamin J. Frisch, … , Michael W. Becker, Laura M. Calvi


In-Press Preview

The bone marrow microenvironment (BMME) contributes to the regulation of hematopoietic stem cell (HSC) function, though its role in age-associated lineage skewing is poorly understood. Here we show that dysfunction of aged marrow macrophages (Mφs) directs HSC platelet-bias. Mφs from the marrow of aged mice and humans exhibited an activated phenotype, with increased expression of inflammatory signals. Aged marrow Mφs also displayed decreased phagocytic function. Senescent neutrophils, typically cleared by marrow Mφs, were markedly increased in aged mice, consistent with functional defects in Mφ phagocytosis and efferocytosis. In aged mice, Interleukin 1B (IL1B) was elevated in the bone marrow and caspase 1 activity, which can process pro-IL1B, was increased in marrow Mφs and neutrophils. Mechanistically, IL1B signaling was necessary and sufficient to induce a platelet bias in HSCs. In young mice, depletion of phagocytic cell populations or loss of the efferocytic receptor Axl expanded platelet-biased HSCs. Our data support a model wherein increased inflammatory signals and decreased phagocytic function of aged marrow Mφs induce the acquisition of platelet bias in aged HSCs. This work highlights the instructive role of Mφs and IL1B in the age-associated lineage-skewing of HSCs, and reveals the therapeutic potential of their manipulation as antigeronic targets.

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Aged marrow macrophages expand platelet-biased hematopoietic stem cells via Interleukin1B

Benjamin J. Frisch\textsuperscript{1,2,*}, Corey M. Hoffman\textsuperscript{1,2,3,*}, Sarah E. Latchney\textsuperscript{1,2}, Mark W. LaMere\textsuperscript{1,2}, Jason Myers\textsuperscript{2,4}, John Ashton\textsuperscript{2,4}, Allison J. Li\textsuperscript{1,2}, Jerry Saunders II\textsuperscript{5,6}, James Palis\textsuperscript{2,5}, Archibald S. Perkins\textsuperscript{2,6}, Amanda McCabe\textsuperscript{7}, Julianne N. Smith\textsuperscript{7}, Kathleen E. McGrath\textsuperscript{5}, Fatima Rivera-Escalera\textsuperscript{8}, Andrew McDavid\textsuperscript{9}, Jane L. Liesveld\textsuperscript{1,2}, Vyacheslav A. Korshunov\textsuperscript{1,10}, Michael R. Elliott\textsuperscript{8}, Katherine C. MacNamara\textsuperscript{7#}, Michael W. Becker\textsuperscript{1,2}, Laura M. Calvi\textsuperscript{1,2#}

\textsuperscript{1}\textit{Department of Medicine}, \textsuperscript{2}\textit{James P. Wilmot Cancer Institute}, \textsuperscript{3}\textit{Department of Pharmacology and Physiology}, \textsuperscript{4}\textit{Genomic Research Center}, \textsuperscript{5}\textit{Center for Pediatric Biomedical Research}, \textsuperscript{6}\textit{Department of Pathology and Laboratory Medicine}, University of Rochester School of Medicine and Dentistry, Rochester NY, \textsuperscript{7}\textit{Department of Immunology and Microbial Disease}, Albany Medical College, Albany, NY, \textsuperscript{8}\textit{Department of Microbiology and Immunology}, \textsuperscript{9}\textit{Department of Biostatistics and Computational Biology}, \textsuperscript{10}\textit{Aab Cardiovascular Research Institute}, University of Rochester School of Medicine and Dentistry, Rochester NY

*These authors contributed equally to the work.

#Corresponding authors:
Laura M. Calvi
Endocrine Metabolism Division
Department of Medicine
University of Rochester Medical Center
School of Medicine and Dentistry
601 Elmwood Ave, Box 693
Rochester, NY 14642
Email: laura_calvi@urmc.rochester.edu
Phone: (585) 275-2901

\textbf{Running Title:} Macrophages and hematopoietic stem cell aging
The bone marrow microenvironment (BMME) contributes to the regulation of hematopoietic stem cell (HSC) function, though its role in age-associated lineage skewing is poorly understood. Here we show that dysfunction of aged marrow macrophages (Mφs) directs HSC platelet-bias. Mφs from the marrow of aged mice and humans exhibited an activated phenotype, with increased expression of inflammatory signals. Aged marrow Mφs also displayed decreased phagocytic function. Senescent neutrophils, typically cleared by marrow Mφs, were markedly increased in aged mice, consistent with functional defects in Mφ phagocytosis and efferocytosis. In aged mice, Interleukin 1B (IL1B) was elevated in the bone marrow and caspase 1 activity, which can process pro-IL1B, was increased in marrow Mφs and neutrophils. Mechanistically, IL1B signaling was necessary and sufficient to induce a platelet bias in HSCs. In young mice, depletion of phagocytic cell populations or loss of the efferocytic receptor Axl expanded platelet-biased HSCs. Our data support a model wherein increased inflammatory signals and decreased phagocytic function of aged marrow Mφs induce the acquisition of platelet bias in aged HSCs. This work highlights the instructive role of Mφs and IL1B in the age-associated lineage-skewing of HSCs, and reveals the therapeutic potential of their manipulation as antigeronic targets.
INTRODUCTION

Dysfunction of the human hematopoietic system with age includes diminished immune response, marrow failure and clonal selection (1). Aging is also associated with a general increase in tissue inflammation that remains largely unexplained (2). The mechanisms driving these characteristics of aged hematopoiesis have, to date, primarily been attributed to intrinsic hematopoietic stem cell (HSC) changes (3). With age, in both humans and mice, the phenotypic long-term HSC (LT-HSC) pool is expanded and globally LT-HSCs differentiate preferentially towards the myeloid lineage (4-6). Multipotent HSCs with platelet bias were recently identified by a number of investigators describing their increased expression of von Willebrand Factor (vWF)(7) and of the Integrin αIIb (CD41)(8, 9). Recent data demonstrate that aged murine HSCs also have increased cell-surface expression of CD41 and vWF (8, 10). Notably, human aged HSCs display platelet (or megakaryocytic) bias (6, 11), suggesting that insights in mechanisms determining murine HSC platelet bias will not only improve our understanding of diseases attributed to the aging hematopoietic system, but also provide novel therapeutic approaches to hematopoietic dysfunction associated with advanced age.

Since the bone marrow microenvironment (BMME) critically regulates HSCs, whether it be considered instructive or enabling distinct HSC fates (12), unique characteristics of the aged BMME could contribute to HSC changes associated with age. In fact, in the Drosophila gonad, extrinsic signals from the niche contribute to stem cell aging(13), and mathematical models have suggested that non-cell-autonomous changes could drive this process in mammalian HSCs (14). While data have suggested that aged endothelial and mesenchymal BMME populations are abnormal and may participate in HSC aging (15-17), microenvironmental signals governing the megakaryocytic bias of aged HSCs remain unclear. Thus, we hypothesized that defects in critical BMME populations caused by age could lead to the expansion of platelet-biased HSCs.

We found that macrophages (Mφs) within the aged BMME could impose the megakaryocytic bias characteristic of aging in HSCs. Aged human and murine marrow Mφs had distinct transcriptional profiles compared to young Mφs, including an increased inflammatory activation signature. We identified increased interleukin 1B (IL1B) mRNA in aged marrow Mφs and elevated caspase 1 activity in Mφs and neutrophils from aged bone marrow. Moreover, IL1B signaling was necessary and sufficient to induce HSC bias and drive young HSPCs to adopt an aged phenotype. While investigating the cause of this increase, we made the novel observation that aged marrow Mφs had a defect in efferocytosis—their ability to clear apoptotic cells. Clearance of apoptotic cells is a critical function of Mφs that prevents necrosis of dead cells and associated local inflammation(18, 19) and also triggers anti-inflammatory responses in phagocytes(20). In young mice, removal of phagocytic cells or genetic loss of the efferocytic receptor Axl increased HSCs with megakaryocytic bias, suggesting that the efferocytic defect in aged marrow Mφs leads to the increase in IL1B activation and signaling. Together these data define a novel mechanism within the aged BMME that enables a specific HSC fate.
RESULTS

Megakaryocytic HSC bias characteristic of aged HSCs is imparted by aged marrow macrophages

Intravital multiphoton microscopy allows for the visualization of the BMME while minimizing artifactual distortion of the marrow (21). In vivo aged (20-24 months old) compared with young (6-8 weeks old) C57Bl/6J mice demonstrated global remodeling of the bone marrow (Figure 1A, B), with decreased bone matrix, while bone marrow cavity and vascular volumes were increased (Figure 1C-E). In aged mice, mature osteoblasts were decreased (Figure 1 F, G), while mesenchymal cell populations in the bone marrow were increased (Figure 1 H, I). Flow cytometric quantification for osteoblastic cells (OBCs), multipotent stromal cells (MSCs) and MSC subsets previously demonstrated to be critical for HSC support, such as PDGFRα+ Sca1+ cells (PaS), PDGFRα/CD51(PaαV) cells and Leptin Receptor+ (LepR+) cells (22-25) confirmed the increase in MSC subpopulations (Figure 1 J-R), also recently reported by others (17). However, though vascular volume was increased (Figure 1C) an increase in bone marrow endothelial cells was not observed (Figure S1). Therefore, the BMME is significantly altered by aging and could contribute to age-induced changes in hematopoiesis. In aged mice, we confirmed that the HSC pool was expanded (Figure S2A-E) and displayed myeloid and megakaryocytic skewing (Figure S2F-I), as was also reported in humans (4-6). We found that the cell surface expression of CD41 was increased not only in aged ST-HSC and LT-HSCs, as previously reported (8, 10), but also on multi-potent progenitor (MPP) populations in aged mice (Figure 2A-G). The increase in megakaryocytic bias of aged HSCs was also supported by an increase in megakaryocytic progenitors (Figure S2H, I). Consistent with these findings, platelet numbers were also significantly increased in the peripheral blood of aged mice, while red blood cells and hemoglobin concentrations were decreased, suggesting that the megakaryocytic skewing was not accompanied by an erythroid bias (Figure S3). To identify additional markers of megakaryocytic bias in aged HSCs, single cell RNA-sequencing of phenotypic LT-HSCs (Lineage− SCA1+ CKIT+ CD150− CD48− FLT3+) from young and aged mice was performed. Principal Component Analysis (PCA) demonstrated segregation of the transcriptional profiles of young compared to aged LT-HSCs (Figure 2H). Greater numbers of aged LT-HSCs displayed high expression of several genes found to be expressed in megakaryocyte (Mk) biased young LT-HSCs (7, 26), including Selp, Vwf and Itgb3 (Figure 2I). The latter, encoding the protein CD61/integrin B3, was also identified in recent global and single cell transcriptional analysis of LT-HSC from young and aged mice (4, 27, 28). Based on these transcriptional profiles, we next identified expansion of CD61+ cells within the Lineage− SCA1+ CKIT+ (LSK) pool of aged mice (Figure 2J). A similar proportion of aged HSCs were also found to be positive for both CD41 and CD61 (Figure 2K). Therefore, cell surface expression of CD41 and CD61 is strongly associated with the aged megakaryocytic HSC bias. To determine if CD41+ cell surface expression on LT-HSCs identifies LT-HSCs with megakaryocytic skewing, we sorted CD41+ or CD41− LT-HSCs from the marrow of aged
mice, and transplanted them in competition with marrow from mice expressing enhanced green fluorescent protein (GFP) under the direction of the human ubiquitin C promoter (*UBC-GFP* mice), in order to quantify LT-HSC donor (GFP+) contribution to platelets. Recipient mice were also *UBC-GFP* (Figure 2L). Percent donor and total platelet contribution was significantly increased in mice transplanted with CD41<sup>+</sup> LT-HSCs (Figure 2M, N). Consistent with increased megakaryocytic skewing, CD41<sup>+</sup> LT-HSCs also demonstrated a statistically significant increase CFU-MK generation in vitro (Figure 2O). Therefore, expression of CD41 by HSCs identifies a population of HSCs with increased megakaryocytic skewing.

The age-dependent in vivo expansion of HSC-supportive MSC subsets was recapitulated ex vivo in cultures of aged BMME cells (Figures 3A, B and S4) that did not show an increase in MSC function (Figure 3C). Therefore, the ability of aged BMMEs to induce the megakaryocytic HSC bias was assessed by co-culturing BMME cells with HSC-enriched LSK cells from young GFP+ mice (Figure 3A). Aged BMME cells significantly expanded the pool of phenotypic megakaryocytic-biased HSCs (Figure 3 D-F). The expanded LSK population included functional HSCs, evidenced by a significant increase in their engraftment from competitive reconstitution assays performed post co-culture (Figure 3G), consistent with the known increase in self-renewal in megakaryocytic-biased HSCs (7, 8, 30). This co-culture however did not induce myeloid bias (Figure S5), also consistent with functional studies of megakaryocytic-biased HSCs (7, 26). This is in contrast with a previously reported decrease in engraftment capacity and myeloid bias of HSPCs co-cultured with aged endothelial cells (31), suggesting that distinct cell components of the BMME differentially impact HSPCs in aged mice. These data support the hypothesis that signals from aged BMME cells can contribute to the megakaryocytic bias of aged HSCs.

We next used a reductionist approach to identify the source within the aged BMME of signals responsible for HSC bias. Since in ex vivo BMME cells there was a large contribution of Mφs (Figure 4A), a population that was also implicated in HSC regulation (32-34), we investigated if Mφs were responsible for the megakaryocytic bias imparted by the aged BMME. Sorted marrow Mφs from young or aged mice (Figure S6) were added to young BMME cells (Figure 4B). The addition of aged marrow Mφs to young BMME cells induced a significant increase in CD41<sup>+</sup> LSKs, similar to that produced by the total aged BMME compartment (Figure 4C). However, adding young Mφs did not increase CD41<sup>+</sup> LSK, suggesting that qualitative rather than quantitative changes in aged BMME Mφs drive the induction of megakaryocytic bias in HSCs, and that aged Mφs are sufficient to induce an aged phenotype even in the presence of young Mφs. The addition of aged BMME Mφs expanded HSC-supportive MSCs (Figure 4D, E), consistent with the BMME changes observed in vivo (Figure 1M-P) and ex vivo (Fig. 3A), suggesting that aged marrow Mφs impact not only HSCs, but also mesenchymal populations known to regulate and support HSCs. Notably, more mature osteoblastic populations were decreased (Figure 4F) with the addition of Mφs from the aged BMME. These results demonstrate that
aged BMME Mφs can induce a megakaryocytic bias in HSCs and an aged phenotype in young mesenchymal BMME cells.

Aged marrow macrophages from both mice and humans have increased inflammatory activation

To identify signals from aged BMME Mφs responsible for inducing the megakaryocytic bias in HSCs, we queried the transcriptional profiles of BMME Mφs from young and aged mice (Figure 5A, B). Gene Ontology (GO) and KEGG Pathways analyses identified an increase in the inflammatory response in aged murine Mφs (Figure 5B), which we confirmed by an increase in phenotypic expression of the major histocompatibility complex II (MHC-II) (Figure 5C) and of CD86 (Figure 5D) in aged BMME Mφs in vivo. This increase in inflammatory activation was also found in our ex vivo system (Figure 5E). Among the pro-inflammatory signals identified in aged murine Mφs, IL1B, previously reported to impact HSC myeloid bias (35), was upregulated in aged BMME Mφs from mice (Figure 5F). We also measured increased IL1B in the marrow of aged mice compared to young mice (Figure 5G). In spite of significant divergence in the human samples (Figure 5H), the increases in immune activation and inflammatory signals were conserved in aged BMME Mφs (Figure S7) from human marrow, as shown by gene enrichment analysis (Figure 5I). Reanalysis of published human microarray data identified transcriptional upregulation of the IL1B receptor (IL1R1) in HSCs from elderly humans compared to young (GSE32719: log2 6.13 vs 4.83, ANOVA p=0.0029)(6). Therefore, IL1B and activation of the IL1R1 are likely be relevant to both murine and human aged HSCs.

IL1B signaling is necessary and sufficient to induce HSC bias imposed by the aged BMME

We next assessed whether IL1R1-dependent signals are necessary and sufficient to mediate induction of platelet bias in HSCs by the BMME. The addition of the IL1R1 antagonist Anakinra to aged BMME cells blocked the increase in CD41+ (Figures 6A, B) and CD61+ (Figure 6C) young LSKs, thus mitigating the effect of the aged BMME on young HSCs. Addition of IL1B to young BMME cells ex vivo could increase numbers CD41+ and CD61+ LSKs (Figures 6D-F). These data demonstrate that IL1R1-mediated signals are required to induce an aged phenotype consistent with megakaryocytic bias in HSCs, and suggest that the microenvironmental signals contributing to HSC aging could be attenuated. Moreover, IL1B is sufficient to initiate megakaryocytic bias in young HSCs.

Potential sources of IL1B in the aged BMME

To elucidate potential sources of mature IL1B in the marrow, we quantified caspase-1 active cells (Figure 7A), since mature IL1B is generated by caspase-1-dependent cleavage of pro-IL1B (36). We found a significant increase in the number of neutrophils and Mφs from aged mice with active caspase-1 (Figure 7B, C), indicating that both populations represent potential sources of IL1B in the
aged BMME. There were no significant changes in key HSC-supportive BMME populations, such as mesenchymal, osteoblastic and endothelial cell populations, with respect to caspase 1 activation in aged mice (Figures 7D-I), suggesting that these BMME populations are not likely sources of the increased IL1B increase in the marrow of aged mice.

**Aged BMME macrophages display defective phagocytosis**

The transcriptome of aged marrow Mφs from mice and humans showed an increase in inflammatory signals (Figure 5). Since it was recently reported that phagocytosis induces an anti-inflammatory program in Mφs (37), we hypothesized that aged marrow Mφs may be defective in their ability to phagocytose. Consistent with this hypothesis, aged marrow Mφs exhibited decreased expression of the key scavenger receptor Mrc1/CD206 (37) (Figure 8A) consistent with reduced cell surface expression of this receptor (Figure 8B), and an overall decrease in the pool of CD206+ Mφs in the marrow of aged mice (Figure 8C). This phenotypic change correlated with a functional disruption of phagocytosis by aged BMME Mφs both in vivo (Figure 8D) and ex vivo (Figure 8E) as measured by the capacity of macrophages to phagocytose fluorescently labeled liposomes.

Next, we tested if loss of phagocytic cells could induce a megakaryocytic bias in the HSCs of young mice. Young mice were treated with clodronate-loaded liposomes (38) in order to deplete phagocytic Mφs (Figure 8F). In vivo treatment with clodronate-loaded liposomes (34, 38) expanded CD41+LT-HSCs (Figures 8G-J), suggesting loss of phagocytic Mφs is sufficient to initiate BMME changes that induce HSC megakaryocytic bias in vivo. This is consistent with observations in a mouse model of aplastic anemia where clodronate-mediated depletion of macrophage induced increased CD41+ LT-HSCs that exhibited robust platelet output in transplantation (39).

**Aged BMME macrophages are defective in efferocytosis of senescent neutrophils**

Neutrophils are short lived and, when senescent, are removed from circulation at daily rates estimated to be $10^7$ in mice and $10^{11}$ in humans (40). Since Mφs remove senescent neutrophils by phagocytosis (40), a phagocytic defect in aged marrow Mφs may increase senescent neutrophils in the circulation and in the marrow. Consistent with the important role of marrow Mφs in the clearance of senescent neutrophils (40, 41) and with our data showing that aged marrow Mφs have phagocytic defects, aged mice had increased total (Figure 9A) and senescent (CXCR4+CD62Llo) neutrophils in the circulation (Figure 9B) and in the bone marrow (Figure 9C), suggesting that marrow neutrophils may also contribute to the increase in marrow IL1B in aged mice.

Marrow Mφs remove and clear senescent neutrophils through efferocytosis, a form of specialized phagocytosis that minimizes the inflammatory consequences of apoptosis (20, 42). Given the phagocytic defects of marrow Mφs and the increase in senescent neutrophils in the marrow of aged mice, we sought to quantify engulfment of senescent neutrophils in young and aged mice (Figures 9D,
E)(43). We demonstrate that uptake of senescent neutrophils is significantly reduced in aged mice supporting our hypothesis that aged Mφs have defective efferocytotic capacity Mφs (Figure 9F).

**Loss of efferocytic receptor Axl is sufficient to drive premature myeloid-megakaryocytic bias in young HSCs**

We next wanted to identify the mechanism responsible for the efferocytic defect in aged marrow Mφs. Efficient efferocytosis requires the activation of specialized tyrosine kinase receptors TYRO3, AXL and MERTK (TAM Receptors), however these receptors may be differentially expressed in distinct phagocytic populations (44). Marrow Mφs from young mice had very low expression of Tyro3, consistent with previous data (44), and this level was not significantly changed in aged marrow Mφs (Figure 10A). The expression of Mertk (Figure 10B) was significantly decreased in aged marrow Mφs, however the cell surface expression of MERTK was not changed in aged marrow Mφs (Figures 10C, D), suggesting that MERTK may not be a critical mediator of the efferocytic defect displayed by aged marrow Mφs. However, Axl expression was significantly downregulated in aged marrow Mφs (Figure 10E). GAS6, the accessory protein that bridges binding of phosphatidyl serine to TAM receptors (45, 46), was previously shown to be required to activate AXL (44), was significantly reduced in the marrow, consistent with reduced transcriptional expression of Gas6 was significantly decreased in aged marrow Mφs, as was its protein level in the marrow of aged mice (Figures 10F, G). Moreover the expression of the efferocytic response gene Abca1 (Figure 10H) (40, 42) was downregulated in aged BMME Mφs, consistent with the observed defective efferocytosis in aged marrow Mφs.

Therefore, we initiated studies with mice lacking the efferocytic receptor Axl, (47) (Axl−/− mice)(48) to determine if specifically blocking efferocytosis could mechanistically explain the age-associated increase in megakaryocytic bias in HSCs that correlates with reduced efferocytosis. Axl−/− mice were reported to have peritoneal Mφs with decreased phagocytic activity (44), and here we demonstrate that marrow Mφs in young Axl−/− mice exhibit defective engulfment of senescent neutrophils (Figure 10I-K). If a defect in efferocytosis is sufficient to induce HSCs to acquire megakaryocytic skewing, young mice with genetic loss of Axl would be expected to have increased CD41+LT-HSCs. Consistent with this, while the size of the LT-HSC pool remained unchanged (Figure 10L), young Axl−/− mice had increased CD41+ (Figure 10M) and CD41+CD61+ (Figure 10N) LT-HSCs, demonstrating that an efferocytic defect induced by loss of Axl is sufficient to induce megakaryocytic bias in young HSCs. LT-HSCs from young Axl−/− mice were superior to those from wildtype littermates in their ability to engraft in competitive transplantation assays (Figure S8), consistent with the functional characteristics of HSCs with megakaryocytic skewing, and with the impact of the aged microenvironment on young HSCs (Figure 3B-G).
DISCUSSION

Our data define a previously unrecognized role for efferocytic defects in tissue-specific Mφs from aged marrow in regulating HSC megakaryocytic bias and provide evidence for the critical function of the BMME as a determinant of age-induced HSC lineage bias. We demonstrate a novel mechanism by which the marrow BMME induces age-dependent changes in HSCs through Mφs that display a pro-inflammatory phenotype and are defective in efferocytosis. Together, our data provide strong evidence that the BMME can indeed induce the Mk bias characteristic of aged HSCs, demonstrating an instructive role of the aged niche that could be broadly applied to the regulation of other tissue-specific stem cell populations. In addition, we demonstrate that the inflammatory signals in murine marrow Mφs are also recapitulated in the human bone marrow, suggesting that the mechanisms identified herein may contribute to the understanding of clonal selection and increased inflammation observed in humans with aging (1, 5, 49).

Recent reports have begun to elucidate the importance of BMME-derived signals in HSC aging, including osteopontin (16), sympathetic nervous system innervation (17) and endothelial cells aging (31). Our work identifies a previously unappreciated role for aged marrow Mφs in orchestrating the specific increase in HSC Mk skewing observed in aging. In defining marrow Mφs as a regulatory component of the HSC niche, our data align with reports suggesting that bone marrow Mφs support both physiological and pathological hematopoiesis (32-34).

While tissue Mφ heterogeneity is well-described (50) even within a specific organ (51), specialization of marrow Mφs with respect to efferocytosis and immune activation during aging is poorly understood. Our data suggest that with aging there is a shift toward an inflammatory M1-like Mφ within the bone marrow, as M1 markers MHCII, CD86, and IL1B production are all increased, and the M2 macrophage marker CD206 is decreased in aged macrophages (52, 53). Conflicting data were previously reported regarding functional phagocytic changes in tissue Mφs with aging, with studies identifying decreased, unchanged, or increased function (54-61), with controversial results even within the bone marrow (62, 63). With respect to phagocytosis, we uncovered significant loss in phagocytic and efferocytic function in BMME Mφs using a physiologically relevant model. Phagocytic defects were recently described in a model of Alzheimer’s disease, a neurodegenerative disorder strongly associated with age. In that model, insufficient generation of phagocytic microglia resulted in their inability to clear amyloid plaques (64). In the context of this report, our data further support the notion that defects in phagocytosis in certain subsets of tissue-based Mφs may be a hallmark of advanced age. Additional data at the single cell level are needed to determine if the functional defect we uncovered in aged BMME Mφs is due to a global phagocytic impairment or to loss of a specific subpopulation of phagocytic Mφs within bone marrow.

The tyrosine kinase receptors MERTK, TYRO3 and AXL are essential for the ability of macrophages to engulf apoptotic cells. Transcriptional analysis of murine marrow macrophages
showed that Tyro3 is expressed at low levels in marrow Mφs and it is not regulated by aging, while both Axl and Mertk expression is decreased with aging. However, flow cytometric analysis of MERTK cell surface expression did not show changes with aging, suggesting that MERTK may not be the critical TAM responsible for the loss of efferocytic capacity of aged marrow Mφs. Loss of Axl in the aging marrow is somewhat surprising given data showing Axl upregulation in the setting of a pro-inflammatory environment (44). In our studies, the decrease in Axl expression observed in aged marrow Mφs may represent an initiating defect that then induces inflammation secondary to the decrease in efferocytic function.

Increased Mk-biased HSCs in mice lacking only one of three TAM receptors that mediate efferocytosis (Axl−/− mice) was also somewhat unexpected since TAM receptors represent a redundant system; thus our findings point to a potentially important role of Axl in bone marrow Mφs. Given the previously reported dependence of Gas6 expression on AXL (44), a finding that is supported by our data, age-dependent decrease in Axl may also decrease transcriptional expression of Gas6, compounding efferocytic defects. In further support of an age-dependent loss of efferocytic function in marrow Mφs, we found age-dependent regulation of Mertk and Abca1, key genes rhythmically modulated in Mφs by circadian changes in clearance of senescent neutrophils (40). While our in vivo studies using Axl−/− mice cannot definitively rule out a potential role for Axl in other cell types, when taken together with our in vitro studies strongly support a role for Mφs in age-induced Mk-bias. Future studies to investigate Axl regulation and function in marrow Mφs are warranted.

The elimination of apoptotic cells is a widely recognized and critical mechanism supporting organ growth and fitness, since apoptosis of cells occurs essentially in all tissues as part of normal development and homeostasis, and lack of apoptotic cell removal contributes to tissue pathology (65). Since it was recently reported that phagocytosis induces an anti-inflammatory program in Mφs (37), we would expect that clodronate-removal of phagocytic macrophages would selectively remove non-inflammatory Mφs. Consequently, senescent neutrophils as well as non-phagocytic Mφs, higher in IL1B production, would remain to cause skewing even of young HSCs in mice treated with liposomal clodronate. This is consistent with our observations using clodronate-loaded liposomes to deplete Mφs in young mice and further supported by the recent demonstration that Mφ depletion in a model of severe aplastic anemia elicits a population of CD41hi HSCs that exhibit robust platelet output (39).

The age-dependent decline in efferocytosis may induce HSC aging through remodeling of additional niche constituents, based on previous data demonstrating the impact of clearance of senescent neutrophils on rhythmic modulation of the HSC niche (40). In support of this, we show that the addition of aged Mφs to young BMME alters specific MSC populations in a manner characteristic of aged BMME. Because restoration of efferocytosis may be targeted (63), our data suggest the potential for remediation of age-induced microenvironmental defects. On the other hand, global inflammation
causes depletion of platelets that may result in significant morbidity (26), thus the efferocytic defect and megakaryocytic HSC bias may be beneficial in the context of aging.

We focused on IL1B as a key inflammatory mediator in the hematopoietic system based on our transcriptional analysis. Recent data also show that chronic pharmacologic dosing of IL1B increases the number of myeloid-primed CD41+ HSCs (35). Moreover, IL1B induces Mk maturation and platelet activation (66), however its role on HSC megakaryocytic skewing was not previously reported. We have demonstrated that aged bone marrow Mφs have increased production of active IL1B. Additionally, defective efferocytosis in aged Mφs leads to increased numbers of senescent neutrophils in the BMME further contributing to increased IL1B concentrations, as senescent neutrophils are a potent source of active IL1B. Thus, defective clearance by marrow macrophages appears to increase IL-1b via two distinct mechanisms. We recapitulated the effect of aged BMME cells with the addition of IL1B in young BMME cells, and demonstrated the critical role of IL1B by blocking the effect of aged marrow microenvironmental cells on HSCs with treatment with an IL1R1 antagonist, pointing to a potential strategy for HSC rejuvenation. Together, the studies presented here identify IL1B as a critical microenvironmental signal necessary and sufficient to induce HSC aging.

Collectively, our findings suggest that the efferocytic defect in aged niche Mφs drives an aged HSC phenotype providing the first mechanistic explanation for megakaryocytic bias seen during aging. Our findings suggest that loss of efferocytosis in aged niche Mφs may increase local IL1B through both increased production of IL1B by aged marrow Mφs and by decreased engulfment of IL1B-rich senesced neutrophils. Since efferocytosis has immunomodulatory roles by engulfing apoptotic cells and by modulating inflammatory signals in Mφs, bringing about resolution of inflammation (42), decreased engagement of efferocytotic signals in Mφs would be expected to enhance their production of IL1B, increasing BMME IL1B. IL1B and activation of the IL1R1 were necessary and sufficient to recapitulate the phenotypic HSC bias, implicating this cytokine as the executor of aged marrow Mφ dysfunction (Figure S9). These data illustrate how, given the emerging heterogeneity of the HSC niche, only complex genetic and biochemical studies of niche HSC interactions could provide novel and more specific therapeutic targets to prevent or reverse not only hematopoietic defects but also the increase in global inflammation associated with advanced age. Our studies provide an important framework to probe the precise mechanisms regulating Mφ function during aging, and open novel therapeutic avenues for rejuvenating aged HSCs.
METHODS

Mice. Male mice were maintained within the Vivarium facility at the University of Rochester School of Medicine and Dentistry. Aged mice (20-30 months) C57B6/J-NIA were obtained from the National Institute on Aging (NIA) aged rodent colonies. Young (8-16 weeks) C57B6/J were purchased from Jackson labs or bred in house. B6.SJL-Ptprca Pepcb/BoyJ mice, which expresses the CD45.1 congenic marker, were bred in house. UBC-GFP mice (C57BL/6-Tg(UBC-GFP)30Scha/J) were purchased from Charles River. Axl-/- were obtained from Dr. Korshunov’s laboratory, were previously described, and have been backcrossed onto the C57bl/6 background for more than 10 generations (48). Treatment with clodronate or PBS liposomes was done as previously described (38).

Complete Blood counts. Blood was collected from the submandibular plexus and collected in EDTA coated tubes. The scil Vet abc Plus+ (Gurnee, IL) was used to determine complete blood counts (CBCs).

Intravital imaging. Mice were injected intravenously with 100µL of 5-10mg/mL 70-kDa-dextran conjugated to Texas Red (Invitrogen) prior to anesthesia. Animals were anesthetized with 5% isoflurane mixed with air which was switched to 1-2% during surgery and subsequent imaging. Calvaria were prepared for in vivo imaging as described (67). Intravital imaging was done using Olympus FV1000-AOM multiphoton system. Fluorescence was collected with an Olympus XLPlanN 25X objective (NA 1.05) and detected with two proprietary external photomultipliers (Hamamatsu).

Image quantification. 70-kDa-dextran and second harmonic generation (SHG) volumes were calculated using Amira 5.4.5. Positive signal was determined by using the threshold feature along each slice. This was further refined by using the smooth and remove island feature. Marrow cavity volume was determined by subtracting the volume of the SHG signal from the entire volume of the z-stack.

Histology. Tissue was prepared as previously described(49). The GBI Polink DS-RRt-Hu/Ms A Kit was used according to the manufacturer’s instructions (Cat# DS211A-60). Endomucin (Cat# 14-5851, ebioscience) was used at 1:500 and Osteocalcin (Cat# ALX-210-333-C100, Enzo Life Science) was used at 1:8000. The GBI Polink-2 HRP-Plus Goat DAB Kit (Cat# D43-110) was used according to the manufacturer’s instruction to detect leptin receptor at 1:1000 (R&D Systems Cat# AF497).

Light microscopy. Images were taken at room temperature using an Olympus BX50 microscope, Olympus DP25 camera, and 100x pan oil immersion objective (NA 1.25). Images were also taken at room temperature on an Olympus BX41, Olympus DP70 camera, and 20x UPlanFl objective (NA 0.5). Cellsens software (Olympus) was used to acquire images on both microscopes.
Flow cytometry. Analysis of marrow cell populations was done as previously described (49) with the following changes. For marrow cell analysis, marrow was released by crushing with a mortar in pestle in 1X PBS with 2% heat inactivated fetal bovine serum (FBS; Hyclone, Lot AWB98615) and 10 units/mL of DNAse (Cat# FERNEN0521, Thermo).

Isolation of peripheral blood mononuclear cells for analysis of senescent neutrophils was done as previously described (40). Analysis for hematopoietic, stromal, and macrophage cell populations were performed as described (38, 49) (Supplemental Tables 1, 2 and Supplemental Figs. 3, 4). The FAM-FLICA™ caspase-1 Assay Kit (ImmunoChemistry Technologies) was used to detect Caspase-1 activity. DAPI was used to discriminate live/dead cells (Cat# D21490, Molecular Probes; final concentration: 2 µg/mL). Samples were run on an LSRII flow cytometer: 3 Laser 355 nm, 488 nm, and 633 nm (BD Biosciences). Analysis was performed using FlowJo v9.6.5 (Treestar). The gating strategy used to identify populations enriched for cells of interest has been previously described (38, 49, 68, 69). Sorting was done on a FACS Aria-II with 405, 488, 532, and 633 nm lasers (BD Bioscience).

**Colony forming unit (CFU) assays.** CFUs from murine marrow cells were performed as previously described (49). Marrow cell populations were plated at 1x10^6 cells per well in six-well plates and grown at 37°C with 5% CO₂ and 2% O₂. For megakaryocyte progenitor assays, FACS-isolated HSC were plated in megacult-collagen media (StemCell Technologies) at a range of 100 to 500 HSC per well and enumerated at 7 days of culture after acetylcholinesterase staining as previously described (70).

**Human samples.** Human bone marrow samples were obtained from healthy volunteers by standard bone marrow aspiration procedures through iliac crest puncture. Human samples from young (<50 years old, YO) and aged (>50 YO) human volunteers were filtered through a cell strainer and washed with PBS. A list of anti-human antibodies used is in Supplemental Table 1 along with the gating strategy for specific populations.

**In vitro cell culture.** Marrow cells were plated at 3x10^6 cells per well, respectively, in six-well plates. Both populations were cultured in α-MEM media with no ascorbic acid, 10% heat inactivated FBS, 1% penicillin and streptomycin, and 50 µM 2-mercaptoethanol. Cells were incubated in 5% CO₂ and 2% O₂. Media was changed on day four to remove non-adherent and dead cells.

For addition of Mφ in cocultures (Fig. 3), 2.1x10^5 F4/80+ Mφ from young and aged marrow cells were sorted by flow cytometry (Supplemental Table 1) and cultured simultaneously with young marrow cells in 5% CO₂ and 2% O₂.

On day 7, 500 Lin-, SCA1+, CKIT+ cells from young (6-12 weeks) UBC-GFP+ mice were sorted by flow cytometry (Supplemental Table 1) and added to the existing stromal cultures with the following recombinant murine cytokines at 25 ng/mL: SCF (Cat# AF-250-03), Flt3-L (Cat# 250-31L), and IL11.
(Cat# 220-11; all from PeproTech). For addition of inflammatory cytokines (25 ng/ml), on day 7 IL1B (Cat# 211-11B), was added to the basal media before plating LSKs. IL1R1 antagonism was done with IL1RA/Anakinra (Amgen) at 1500 ng/ml added on day 7. Cells were incubated undisturbed for an additional four days in 5% CO₂ and 2% O₂. On day 11, floating and adherent cells were collected for flow cytometry analysis. Adherent cells were removed by incubating cells with 1X TrypLE (Cat# 12605-010, Invitrogen). Floating and adherent cells were also collected on day 7 for phenotyping of stromal monolayers.

**Competitive repopulation assay.** Recipient mice were given 10Gy of radiation, split in two 5Gy doses separated by 24 hours, as previously described (49). For transplant of LSK co-cultures, cells from one well (floating and adherent) were mixed with 300,000 whole bone marrow cells from a CD45.1 competitor. In this scenario, residual hematopoietic cells from the co-culture were CD45.1, the competitor CD45.1, and the donor LSKs were GFP+, allowing us to distinguish all three populations.

For competitive bone marrow transplant of Axl⁻/⁻, 1x10⁶ of both donor and competitor were transplanted. Donor cells expressed CD45.2, the competitor was CD45.1/CD45.2 and the recipients were CD45.1, allowing all three groups to be distinguished.

For analysis of platelet reconstitution, CD41+ and CD41⁻ donor LT-HSCs (100 Lineage-Flt3-CD48-SCA1+CKIT+CD150+ cells per recipient mouse) were sorted from aged mice and combined with 1x10⁶ competitor bone marrow harvested from UBC-GFP mice that express GFP in all tissues including platelets. Donor/competitor cells were transplanted into UBC-GFP recipient mice that were given 10Gy of radiation, split in two 5Gy doses. Proportions of GFP+ and GFP⁻ platelets were determined by flow cytometry on whole peripheral blood sampled 3 weeks after transplantation and stained with anti-CD41 and Ter119 antibodies (Ebioscience) and analyzed on a LSRII (BD Bioscience) using FlowJo analysis software (Treestar).

**RNA isolation and generation of cDNA library.** Mφs were FACS sorted directly in RLT plus buffer (Qiagen). RNA extraction was performed with Qiagen Rn easy PLUS Micro kit following standard operating procedures. RNA quality was assessed using Agilent Bioanalyzer 2100. 1 nanogram of high quality (RIN>8.0) total RNA from each sample was reverse transcribed into cDNA using the Clontech SMART-Seq® v4 Ultra® Low Input RNA Kit. Final Illumina libraries were constructed using 150pg of cDNA with the illumina Nextera XT DNA Library Preparation Kit. Expression datasets are available in GEO Accession GSE100907.

**Transcriptional Analysis of Macrophage Populations.** Raw reads generated from the Illumina HiSeq2500 sequencer were demultiplexed using configurebcl2fastq.pl version 1.8.4. Quality filtering
and adapter removal are performed using Trimmomatic version 0.32 with the following parameters:

"SLIDINGWINDOW:4:20 TRAILING:13 LEADING:13 ILLUMINACLIP:adapters.fasta:2:30:10 MINLEN:15" (71). Processed/cleaned reads were then mapped to the reference genome (Mouse - GRCm38.p4, Human – GRCh38.p3) using STAR_2.4.2a with the following parameters: "--twopassMode Basic --runMode alignReads --outSAMtype BAM SortedByCoordinate --outSAMstrandField intronMotif --outFilterIntronMotifs RemoveNoncanonical --outReadsUnmapped Fastx" (72). Gene-level read quantification was derived using htseq-count 0.6.1 with a GTF annotation file (Mouse – Gencode M6, Human – Gencode 23) and the following parameters: "-q -f bam -s no -r pos -i gene_name" (73). Differential expression analysis was performed using DESeq2 -1.12.4 with an adjusted p-value threshold of 0.05 within R version 3.3.0 (74). Heatmaps were created within R using the pheatmap package (R package version 0.7.7) given rLog transformed expression values.

**Single-cell RNA Sequencing.** Single long-term hematopoietic stem cells (LT-HSC) were captured on a 5–10 µm cell diameter integrated microfluidic chip (IFC) using the Fluidigm C1 Single-cell AutoPrep system (Fluidigm Corporation). Cells were pre-stained with Calcein AM/EthD-1 LIVE/DEAD cell viability assay (Life Technologies) and loaded onto the IFC at a concentration of 500–700 cells/ul. Viable single cell confirmation was performed with phase-contrast fluorescence microscopy to assess the number and viability of cells per capture site. Only single, live cells were included in the analysis. For RNA-seq analysis, cDNAs were prepared “on-IFC” using the SMARTer Ultra Low RNA kit for Illumina (Clontech) following fluidigm recommendations. Single-cell cDNA size distribution and concentration was assessed with PicoGreen (Life Technologies) and Agilent Bioanalyzer 2100 analysis (Agilent Technologies). Illumina libraries were constructed in 96-well plates using Illumina’s NexteraXT DNA Sample Preparation kit following the protocol supplied by Fluidigm. For each C1 experiment, a bulk RNA control and a negative control were processed in parallel, using the same reagent mixes as used on chip. Libraries were quantified by Agilent Bioanalyzer, using High Sensitivity DNA analysis kit, and also fluorometrically, using Qubit dsDNA HS Assay kits and a Qubit 2.0 Fluorometer (Technologies).

**Analysis of Single-cell RNA Sequencing.** Read data were cleaned, aligned, and quantified in the same manner as the mouse RNA-Seq data. The R package scater_1.2.0 was used to load the data into R version 3.3.2 and perform initial filtering of genes (> 0 reads in more than 5 cells) and cells (> 0.5M total reads per cell)) (75). The scran_1.2.2 R package was used to compute sum factors with sizes 20, 40, and 60 prior to normalization with scater “normalize” function (75). The top 1000 highly variable genes were determined using the scran “trendVar” function with use.spikes=FALSE and “decomposeVar”. The R package Seurat_1.4.0.9 was then used to visualize the expression using the “VlnPlot” function and perform principle components analysis using the highly variable genes determined using scran (76).
GO and KEGG analysis. The open source Enrichr gene list enrichment analysis tool (77) was used for Gene Ontology (GO) and KEGG Pathway analysis. Significantly (padj<0.05) differentially regulated genes were selected for analysis when the Log2FoldChange <-1 (downregulated, 508 genes) and >1 (upregulated, 570 genes). Gene annotation was performed within the GO terms “Biological Process/Direct” and KEGG Pathways Analysis using a threshold count 2 and EASE 0.1. Enrichment values were displayed as fold enrichment for terms with FDR<0.05.

Gene Set Enrichment Analysis (GSEA). Overlaps for selected genes were computed using Gene Set Enrichment Analysis (78). Significantly (padj<0.05) differentially upregulated genes were selected for analysis (63 genes). Gene annotation was performed within the GO terms “Biological Process/Direct”. Top hits based on FDR (smallest to largest) were displayed as fold enrichment for terms with FDR<0.05. Fold enrichment was calculated as the ratio of the two proportions.

ELISA. Sera and total bone marrow protein was collected from aged mice. Bone marrow cell lysates were homogenized with a pestle in a buffer containing IGEPAL CA-630 and proteinase inhibitors for protein analysis. ELISA for marrow IL1B and GAS6 were performed in marrow serum using the commercially available kits Mouse IL-1β ELISA kit (ab197742) and Mouse GAS6 ELISA Kit (ab155447) respectively.

Phagocytosis assays. For liposome uptake experiments, young and aged marrow cells were incubated with DOPC/CHOL Liposomes labeled with fluorescein-DHPE dye (FormuMax Scientific, Sunnyvale, CA) for 1 h. Cells were washed and stained for flow cytometric analysis. For in vivo neutrophil uptake experiments, phagocytosis was quantified via flow cytometry as described previously (79). Neutrophils were purified from young marrow cells using the EasySep Mouse Neutrophil Enrichment Kit (StemCell Technologies, Cambridge, MA), following the ‘The Big Easy’ protocol. Neutrophils were grown overnight at 37°C/5% CO₂, labeled with efluor670 (Cat# 65-0840-85, eBioscience), then retro-orbitally injected into young or aged mice, or into young (9-12 weeks old) Axl⁻/⁻ and wildtype (wt) littermates. 18 hours post injection, marrow cells were harvested for flow cytometric analysis.

Statistical analysis. All data are presented as mean ± s.e.m. All analyses were made with GraphPad Prism software (version 5) using 2-tailed Student’s t-test, Mann-Whitney non-parametric testing, 1way or 2way ANOVA with Tukey’s multiple comparison post-test when appropriate. A P value less than 0.05 was considered significant.
**Study approval.** All murine studies were performed in accordance with protocols approved by the institutional animal care and use committee, the University Committee on Animal Resources (University of Rochester, Rochester, NY). Written informed consent was obtained from all human subjects. The study was approved by the institutional review board, the Research Subjects Review Board (University of Rochester, Rochester, NY) and conducted in accordance with the Declaration of Helsinki.

**Accession Codes.** GSE100907

**Author Contributions:** B.J.F., C.M.H., and S.E.L. designed and performed experiments, analyzed data, and wrote the manuscript. M.W.L., K. E. M., and A.L. performed experiments. J.M. and J.A. performed transcriptional analyses. A.M., J.S. and F. R.-E. performed myeloid analysis. J.L.L. provided human specimens. J.P., A.S.P. and V.A.K. participated in study design. A. M. provided statistical consultation. M.R.E contributed to study design and data analysis and performed experiments. M.W.B. contributed to study design and performed experiments. K.C.M. contributed to study design and data analysis. L.M.C. designed and performed experiments, analyzed data, and wrote the manuscript.

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**COMPETING FINANCIAL INTERESTS**
The authors declare no competing financial interests.

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Figure 1: Aged mice have remodeling of their bone marrow and expansion of MSC populations. 

(A,B) Representative images of 2-Photon intravital microscopy of calvaria; second harmonic generation (collagen; blue) and vasculature (70-kDa-dextran; red). Scale bar, 100µm. (C-E) Vascular (A), marrow (B) and collagen (C) volume (N=5-7 mice/group). (F,G) Immunohistochemistry of osteocalcin (pink) and endomucin (brown), scale bar, 10µm. Black arrows indicate osteocalcin positive osteoblasts. (H,I) Immunohistochemistry for LEPR⁺ cells (brown), scale bar, 100µm. Black arrows indicate LEPR⁺ perivascular cells. (J-R) Representative flow cytometry plots (J,M,O,Q) and analysis quantification (K,L,N,P,R) of marrow osteoblastic cells (OBC: CD45TER119⁺CD31⁺CD51⁻SCA1⁻ cells, (J,K)), marrow multipotent stromal cell populations (MSC: CD45TER119⁺CD31⁺CD51⁻SCA1⁺ cells, (J,L)), PaS (CD45⁻TER119⁻CD31⁻CD140a⁻SCA1⁺ cells, (M,N)), PadV (CD45TER119⁺CD31⁻CD140a⁻CD51⁻ cells, (O,P)) (N=13-14 mice/group), and Leptin Receptor positive (LEPR⁺) (CD45TER119⁺CD31⁻LEPR⁺ cells, (Q,R)) (N=9 mice/group). Each symbol represents an individual mouse, data represent mean ± SEM. p-values: two-tailed Student’s t-test; **p<0.01, ***p<0.001.
Figure 2: Aged mice have expanded megakaryocyte biased HSCs.

(A-G) Representative flow cytometry of CD41 expression and its quantification across LSK subsets in young and aged mice N= 6 mice/group. (H) PCA plot of single LT-HSCs from young and aged mice. Variance of each PCA is shown. (I) Violin plots of Selp (ANOVA p=4.94E⁻⁷), Vwf (p=0.003051854) and Itgb3 (p=6.38E⁻⁵) expression from young and aged LT-HSCs N= 76 young HSCs and 63 aged HSCs. (J,K) Quantification of CD61 (N=9 mice/group) and of CD41/CD61 (N = 5 mice/group) expression across LSK subsets in young and aged mice. (L) Schematic representation of experimental design for analysis of megakaryocytic skewing potential of CD41+ LT-HSCs. (M,N) Donor platelet bias of sorted CD41⁺ (N=5 recipients) vs CD41⁻ (N=4 recipients) LT-HSCs as percent donor and total number. (O) Quantification of in vitro CFU-Megakaryocyte colony forming units (CFU-MK) per 100 sorted CD41⁺ vs CD41⁻ LT-HSCs (N=4/group). (C,F,G,L,J,K). Each symbol represents an individual mouse, data represent mean ± SEM. p-values: two-tailed Student’s t-test except as noted; *p<0.05, **p<0.01, ***p<0.001, ns= not significant.
Figure 3: Dysfunction in aged MSCs and regulation of HSCs by aged microenvironments.

(A) Schematic for in vitro co-culture of HSCs and stromal cells. (B) Quantification of PaS cells from in vitro cultures derived young and aged mice (N= 8 wells/group). (C) Quantification of colony forming unit-fibroblast (CFU-F) from young and aged mice N=5 young and aged mice. (D) Quantification of CD41, (E) CD61, and (F) CD41/CD61 expression in LSKs grown on young or aged BMME cells (N=12 young and aged/well). (G) Engraftment of HSCs grown on either young or aged BMME cells (N= 5 recipients/group). (B-F) Each symbol represents an individual mouse, data represent mean ± SEM: (B-F), two-tailed Student’s t-test; *p<0.05, **p<0.01, ****p<0.0001, (G), 2-way ANOVA **p<0.01 between young and aged longitudinally.
Figure 4: Aged macrophages recapitulate age-related changes to HSCs and the BMME in vitro.

(A) F4/80+ cells in BMME cultures derived from young and aged marrow N=14 wells/group. (B) Schematic for addition of sorted Mφs to BMME co-culture. (C) Expression of CD41 on GFP+ young LSK after co-culture with BMME cells with or without young or aged Mφs (N= 6 wells/group). (D-F) Quantification of PaαS cells (D), PaαV cells (E) and OBCs (F) as defined in Figure 1 (N=6 wells/group). Each symbol represents an individual mouse, data represent mean ± SEM: p-values: (A) two-tailed Student’s t-test;***p<0.001, (C-F) 1-way ANOVA with Tukey’s multiple comparison post-test; *p<0.05, **p<0.01, ***p<0.001.
Figure 5: Aged marrow macrophages have a pro-inflammatory phenotype and elevated IL-1β expression.

(A) PCA plot of young (red) and aged (black) marrow Mφs isolated as shown in Supplemental Figure 3 (N=3 mice/group). Variance of each PCA is shown on the axes. (B) Upregulated GO and KEGG categories in aged Mφs compared young (N=3 mice/group). See also Supplemental Tables 3 and 4 for details. (C) Expression of MHC-II and (D) CD86 on Mφs from young and aged mice (N= 5-10 mice/group). (E) Expression of MHC-II in Mφ from BMME in vitro cultures (N= 11-14 well/group). (F) Expression of IL1β in young and aged Mφs (N= 3 mice/group). (G) Quantiﬁcation by ELISA of IL1B protein in marrow of young and aged mice (N=5/group). (H) PCA plot of young (red, N=2) and aged (black N=3) human marrow Mφs isolated as shown in Supplemental Figure 4. Variance of each PCA is shown on the axes. (I) Upregulated GO processes in aged human Mφs compared to young (N=2,3 human marrow/group). Each symbol represents an individual mouse, data represent mean ± SEM: p-values: two-tailed Student’s t-test; *p<0.05, ***p<0.001.
Figure 6: Exposure to IL1B increases megakaryocytic HSCs ex vivo.

(A) Schematic of co-culture LSKs and young or aged stromal cells with anakinra (rhIL1RA). (B) Expression of CD41 and (C) CD61 on LSKs grown in vitro with young or aged BMME cells with or without anakinra (N=3-6 wells/group). (D) Schematic of co-culture LSKs and young or aged stromal cells with recombinant IL-1β. (E) Expression of CD41 and (F) CD61 in LSKs grown in vitro young BMME cultures with IL-1β (N= 18 wells/group). Each symbol represents an individual mouse, data represent mean ± SEM: (B,C) 1-way ANOVA with Tukey’s multiple comparison post-test; *p<0.05, **p<0.01, ***p<0.001; (E,F) two-tailed Student’s t-test; ***p<0.001.
Figure 7: Increased Caspase-1 activity in marrow Mφs and neutrophils but not in other BMME populations in aged mice.
(A) Representative flow cytometry of FAM-FLICA (active Caspase-1) (blue) stained bone marrow cells, and FAM-FLICA+ gate (yellow), overlaid onto a FAM-FLICA FMO control (red). (B,C) Number of marrow Mφs and neutrophils with Caspase-1 activity from young and aged animals (N= 3 mice/group). (D-I) number of microenvironmental cells with Caspase-1 activity from young and aged animals.
Populations quantified include (D) arteriolar endothelial cells (CD45-TER119-CD31+SCA1+), (E) sinusoidal endothelial cells (CD45-TER119-CD31+SCA1+), (F) Pa/αV cells (CD45-TER119-CD140a+CD51+), (G) MSCs (CD45-TER119-CD31-CD51+ SCA1+), (H) PaS cells (CD45-TER119-CD31-CD140a+SCA1+), and (I) OBCs (CD45-TER119-CD31-CD51+ SCA1-) (N=3 mice/group). Each symbol represents an individual mouse, data represent mean ± SEM: p-values: two-tailed Student’s t-test; *p<0.05, ***p<0.001.
Figure 8: Aged Marrow Mφs have defective phagocytosis and loss of macrophages is sufficient to drive premature megakaryocytic skewing of HSCs in vivo.

(A) Expression of scavenger receptor *Mrc1* in young and aged marrow Mφs (N= 3 mice/group). (B,C) Quantification of cell surface expression of MRC/CD206 by mean fluorescence intensity (MFI) (B) and percent of marrow Mφs (C) in marrow from young and aged mice (N=6 mice/group). (D) Quantification of liposome uptake by marrow Mφs (N= 6 wells/group). (E) Quantification of liposome uptake by Mφs in BMME cultures (N=6 wells/group). (F) Schematic of clodronate liposome administration and bone marrow analysis. (G) LT-HSC frequency, (H) CD41 expression, (I) total number, and (J) total CD41+ 14 hours after clodronate treatment. (N= 5-8 mice/group). Each symbol represents an individual mouse, data represent mean ± SEM; p-values: two-tailed Student’s t-test; *p<0.05, **p<0.01, ***p<0.001.
Figure 9: Impaired efferocytosis by macrophages in the bone marrow of aged mice.

(A) Quantification of peripheral blood granulocytes (N= 15 mice/group). (B,C) Quantification of senescent peripheral blood (B, N= 15 mice/group) and marrow (C, N=5 mice/group) neutrophils. (D) Representative flow cytometry plots of young and aged marrow for the identification of Mφs that have phagocytosed senescent neutrophils. (E) Schematic of senescent neutrophil uptake experiment. (F) Quantification of senescent neutrophil engulfment in vivo by marrow Mφs 18 hours post-injection (N= 4 mice/group). Each symbol represents an individual mouse, data represent mean ± SEM: p-values: two-tailed Student’s t-test; *p<0.05, ***p<0.001.
Figure 10: Genetic loss of efferocytic capacity is sufficient to drive premature aging phenotypes.

(A,B) Expression of the efferocytosis genes Tyro3 and MerTK in young and aged marrow Mφs (N= 3 mice/group). (C,D) Quantification of MERTK flow cytometric data, MFI (C) and percent (D) of marrow Mφs. (E,F) Expression of the efferocytosis genes Axl and Gas6 in young and aged marrow Mφs (N= 3 mice/group). (G) Quantification by ELISA of the serum protein level for Gas6 (N= 4 young, 5 aged mice) in young compared to aged murine marrow. (H) Expression of Abca1 in young and aged marrow Mφs (N= 3 mice/group). (I-K) Schematic representation (I) and quantification (J,K) of efferocytic capacity of marrow Mφs from young wt and Axl-/- littermates 18 hours post-injection, shown as percent positive macrophages (J) and MFI (K) (N=3 mice/ experimental group). Quantification of (L) total LT-HSCs, (M) CD41+ and (N) CD41+CD61+ LT-HSCs from WT and Axl-/- mice (N= 9-11 mice/group). (A-N) Each symbol represents an individual mouse, data represent mean ± SEM; p-values: two-tailed Student’s t-test; *p<0.05, **p<0.01 ***p<0.001.