Identification of a Monocyte-Predisposed Hierarchy of Hematopoietic Progenitor Cells in the Adventitia of Postnatal Murine Aorta

Running title: Psaltis et al.; Hematopoietic potential of the postnatal aorta

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Abstract:

**Background** - Hematopoiesis originates from the dorsal aorta during embryogenesis. While adult blood vessels harbor progenitor populations for endothelial and smooth muscle cells, it is not known if they contain hematopoietic progenitor (HPCs) or stem cells (HSCs). Here, we hypothesized that the arterial wall is a source of HPCs and HSCs in postnatal life.

**Methods and Results** - Single cell aortic disaggregates were prepared from adult chow-fed C57BL/6 and *ApoE*−/− mice. In short- and long-term methylcellulose-based culture, aortic cells generated a broad spectrum of multipotent and lineage-specific hematopoietic colony-forming units (CFUs), with preponderance of macrophage CFUs (CFU-M). This clonogenicity was higher in lesion-free *ApoE*−/− mice and primarily localized to Sca-1+ cells in the adventitia. Expression of Sca-1 in the aorta co-localized with canonical HSC markers, as well as CD45 and mature leukocyte antigens. Adoptive transfer of labeled aortic cells from GFP transgenic donors to irradiated C57BL/6 recipients confirmed content of rare HSCs (1 per 4,000,000 cells), capable of self-renewal and durable, low level reconstitution of leukocytes. Moreover, the predominance of long-term macrophage precursors was evident by late recovery of GFP+ colonies from recipient bone marrow and spleen that were exclusively CFU-M. Although trafficking from bone marrow was shown to replenish some of the aorta's hematopoietic potential following irradiation, the majority of macrophage precursors appeared to arise locally, suggesting long-term residence in the vessel wall.

**Conclusions** - The postnatal murine aorta contains rare multipotent HPCs/HSCs and is selectively enriched with Sca-1+ monocyte/macrophage precursors. These populations may represent novel, local vascular sources of inflammatory cells.

**Key words:** vasculature; blood cells; stem cells; atherosclerosis
Cells of hematopoietic lineage play key regulatory roles in a diverse spectrum of vascular diseases, most notably atherosclerosis\(^1,2\). Defining the tissue origins of these cells is crucial to understanding vascular physiology and pathological remodeling. Historically, the longstanding dogma of atherosclerosis has been that mature inflammatory cells are recruited from the peripheral circulation to the expanding plaque\(^1\). However, several lines of evidence prompt consideration of an alternative view that the vasculature itself may harbor resident progenitor cells that act as a source of locally-derived hematopoietic cells throughout adult life.

During embryogenesis, definitive hematopoiesis has a conserved origin across various species within the developing aorta-gonad-mesonephros region and specifically the hemogenic endothelium of the ventral floor of the dorsal aorta\(^3-6\). Although the hematopoietic potential of the embryonic vasculature is thought to be transient\(^6\), it is notable that hematopoietic stem cells (HSCs) and progenitor cells (HPCs) continue to occupy perivascular niches in postnatal bone marrow (BM) and extramedullary hematopoietic tissues\(^7\). Presumably this enduring anatomical connection with the vasculature facilitates their circulation and trafficking to other tissues\(^8-10\).

Traditional paradigms of the postnatal vasculature as a terminally differentiated tissue system have been reconsidered following important findings showing that the adult vessel wall contains reservoirs of diverse stem and progenitor cell populations\(^11\). Evidence now exists for the presence of vascular-resident ancestral cells, distributed throughout the different mural layers of blood vessels, that include intimal endothelial progenitor cells\(^12\), medial side population cells\(^13\), adventitial Sca-1\(^+\) (stem cell antigen-1\(^+\))\(^14,15\) and CD34\(^+\) cells\(^16\) and multipotent pericytes\(^17,18\). Collectively, these cells have been shown to be responsible for progeny of endothelial, smooth
muscle and mesenchymal lineage that may each participate in vessel wall remodeling and postnatal vasculogenesis. In contrast to these progenitor cells, there is a lack of data specifically addressing whether bona fide HPCs and HSCs may also co-exist within the adult vasculature.  

The current study investigated the presence of multipotent HPCs and HSCs within the macrovasculature of adult C57BL/6 mice and pro-atherogenic apolipoprotein E-null (ApoE−/−) mice. Murine aortic disaggregates were found to possess short- and long-term clonogenicity for a broad array of hematopoietic subtypes and achieved durable but low frequency reconstitution of leukocytes after adoptive transfer into lethally irradiated recipients. This hematopoietic capacity is heavily predilected toward monocytes/macrophages and is largely contained within a subpopulation of adventitial Sca-1+ cells. Although trafficking from BM to the adventitia may account for some of its hematopoietic potential, the majority of macrophage precursors appear to be constitutive or long-term residents in the vessel wall, suggesting that the adventitial microenvironment imparts lineage specificity. These findings provide new evidence suggesting a local origin for HPCs in the adult vasculature.

Methods
Detailed methods are provided in the online supporting information. Single cell aortic disaggregates were prepared from 12-24 week-old C57BL/6 and chow-fed ApoE−/− mice. Short- and long-term methylcellulose culture was used to assess the hematopoietic clonal capacity of aortic cells in vitro, with comparisons made to cells from BM, peripheral blood and skeletal muscle. Aortic cells were phenotypically profiled by flow cytometry for expression of canonical HSC and mature leukocyte antigens, while intact vessels were immunostained and imaged under
confocal microscopy. Adoptive transfer of aortic cell suspensions was performed from green fluorescent protein (GFP) transgenic or ROSA26 mice into irradiated C57BL/6 recipients to determine their potential for multilineage hematopoietic reconstitution. The tissue origin of vascular HPCs/HSCs was investigated by tracking engraftment of GFP BM cells in the aortic wall of lethally irradiated C57BL/6 and ApoE−/− mice and their contribution to myelopoietic colony-forming units (CFUs). All animal experiments complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md) and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

**Statistical Analysis**

Data were checked for normality of distribution by D’Agostino-Pearson omnibus test. Inter-group statistical comparisons were performed with parametric or non-parametric two-sample t-tests or ANOVA (with post-test comparisons), as appropriate. For comparisons between different tissue types harvested from the same mice, linear mixed effects models were used. Time-course data were analyzed by one-way or two-way ANOVA, with repeated measures where applicable. Results are expressed as mean±standard error (SEM) of multiple experiments, with significance established at P<0.05.

**Results**

**Aortic cells generate diverse hematopoietic colonies, especially CFU-M**

Complementary *in vitro* and *in vivo* strategies were used to determine whether multipotent HPCs and HSCs exist within macrovessels, using murine aorta as the main source of vascular tissue.
Aortic explants were cleaned by separating all perivascular adipose tissue and thorough flushing to remove peripheral blood cells. Following chemical digestion, filtration and repeated washes, aortic cell pellets were white with negligible hemoglobin content (Supplemental Figure 1). Initial screening for the hematopoietic potential of aortic disaggregates was undertaken with in vitro CFU assays. In short-term methylcellulose culture, aortic cells from C57BL/6 mice generated all types of myelopoietic CFUs, as well as burst-forming units-erythroid (BFU-E) (Figure 1A,B). Rare mixed granulocyte-macrophage colonies (CFU-GM) and multipotent CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) were generated from approximately 40% of experiments and appeared during the first week of culture. In contrast, lineage-specific granulocytic (CFU-G) and macrophage (CFU-M) colonies typically arose between days 10 and 14. CFU-M showed a highly consistent predominance amongst the different colony types (Figure 1B) and consisted of cells of uniform shape and size but variable cell number, ranging from 30-100 cells to large colonies that contained more than 500 cells. These were cultured with similar frequency from different segments of aorta (Supplemental Figure 2A). Macrophage colonies, but not other CFU types, were also generated from disaggregates obtained from carotid and femoral arteries, although there was a trend for reduced yield compared to aorta (Supplemental Figure 2B). Notably, recovery of CFU-M was extremely rare from renal artery disaggregates.

The overall clonogenicity of cells from aorta was lower than that of BM but three-fold higher than for blood (total CFU yield: aorta 14.5±2.0, BM 172±26, blood 4.8±1.6 per 10^5 cells, P<0.0001), with markedly different CFU profiles. The strong predilection for CFU-M was unique to aorta, with much higher frequency than from blood (Figure 1C), while CFU-GM and -
GEMM were 1000-fold and 70-fold more frequent from BM than aortic cultures, respectively (Figure 1D). In light of previous isolation of HSCs from skeletal muscle, it was noteworthy that CFU yield was almost five times more from aortic cells than donor-matched, freshly-isolated skeletal muscle cells (12.6±2.6 vs 2.8±1.4 per 10^5 cells, P<0.01, n=4).

To begin to consider how this vascular hematopoietic potential might be relevant to inflammatory cell accumulation in pro-atherogenic vessels, CFU assays were also performed from chow-fed ApoE^{-/-} mice (total cholesterol 15.4±0.7 mmol/L vs 1.9±0.2 mmol/L for C57BL/6, P<0.0001, n=7 per strain). These were assessed as being lesion-free at the time of aortic harvest to ensure that CFUs were not the progeny of inflammatory cell precursors in established atheroma. Aortic disaggregates from ApoE^{-/-} mice formed almost twice as many CFU-M as age-matched C57BL/6 counterparts, without significant difference for other colony types (Figure 1E, F).

Short-term CFU results were corroborated with long-term culture-initiating cell assays, performed on a BM stromal cell layer. The overall yield of long-term CFUs from C57BL/6 aortas was 1 in 59,200±7,600 cells (n=3), with a preponderance of CFU-M (1 in 73,800±18,300). This compares to an expected CFU yield of 1 in 30,000 from C57BL/6 BM cells (reference manual for StemCell Technologies, Inc., Vancouver, Canada). Furthermore, there was a trend for higher frequency of long-term CFUs from ApoE^{-/-} aortas (1 in 40,400±6,800, n=3, P=0.09 vs C57BL/6 aortas), primarily as a result of greater recovery of CFU-M (1 in 45,300±6,700, P=0.06).
Together these results demonstrate the presence of short- and long-term hematopoietic progenitors throughout the aortic length, with a uniquely predisposed potency for CFU-M. Although CFU-M precursors exist in other arterial territories, their frequency is not uniformly distributed throughout the macrovasculature. Moreover, they are upregulated in ApoE<sup>-/-</sup> mice even in the absence of Western diet and before the apparent onset of intimal lesions.

**Aortic cells express HSC markers**

Detailed immunophenotypic characterization has enabled the delineation of a hierarchy of ancestral hematopoietic cells in BM<sup>21, 22</sup>. Extensive profiling was therefore undertaken on aortic cells with multicolor flow cytometry, focusing on mature lineage antigens and well-described progenitor cell markers (Table 1). Aortic isolates contained populations of mature myelomonocytic cells (Gr1<sup>+</sup>, CD11b<sup>+</sup>, Ly6G<sup>+</sup>, Ly6C<sup>+</sup>) and lymphocytes (CD5<sup>+</sup>, CD45R<sup>+</sup>), consistent with previous data showing the presence of leukocytes in normal aortas<sup>23</sup>. Sca-1, which has been shown to identify smooth muscle progenitors in vascular adventitia<sup>14</sup>, was commonly expressed in the aorta (Figure 2A) and at higher fluorescence intensity than in cells from blood or BM (Supplemental Figure 3A). Smaller populations of aortic cells were positive for the progenitor markers CD34 and c-kit (Supplemental Figure 3B) and displayed phenotypes characteristic of HSCs in BM [KLS<sup>+</sup> (c-kit<sup>+</sup>Lineage<sup>-</sup>Sca-1<sup>-</sup>), KLS<sup>+</sup>CD34<sup>-</sup> and KLS<sup>+</sup>CD150<sup>-</sup>], (Figure 2B). Expression of CD143 (angiotensin-converting enzyme, ACE) which has more recently been associated with hematopoietic primitiveness<sup>24</sup>, was also identified (4.2±1.1% of all C57BL/6 aortic cells, n=8). C57BL/6 and ApoE<sup>-/-</sup> aortas exhibited similar frequencies for these markers, with the striking exception of Sca-1 which was upregulated in the pro-atherogenic mice (Figure 2A; Supplemental Table 1).
Aortic Sca-1+ cells express monocytic markers

Focusing on aortic Sca-1+ cells, there was evidence for considerable immunophenotypic heterogeneity. In addition to shared expression with c-kit and CD34, a proportion of Sca-1 was co-expressed with the pan-leukocyte antigen, CD45 (Figure 2C). Amongst the different leukocyte markers associated with Sca-1+CD45+ cells, the most frequent was Ly-6C, which previously has been shown to define circulating, pro-inflammatory monocytes 25 (Figure 2D). Sca-1+Ly-6C+ cells were more prevalent in aorta than BM (Table 1). Furthermore, as a unique feature of the aorta, Ly-6C was predominantly expressed within the Sca-1+ population, especially in ApoE-/- mice, unlike its expression in BM and blood where it was mostly restricted to Sca-1- cells (Figure 2E,F). On account of the preponderance of CFU-M from aortic isolates, the macrophage colony-stimulating factor (M-CSF)/c-fms signaling pathway was also analyzed. The c-fms receptor (CD115) was expressed rarely on aortic cells from C57BL/6 mice (<0.5%, n=4) but was more common in chow-fed ApoE-/- mice (2.8±1.1%, range 1.8-4.5%, n=4, P<0.05). As with Ly-6C, CD115 was mostly found on Sca-1+ cells in the aorta, differing from BM and blood where >90% of its expression occurred on Sca-1- cells (Figure 2G).

Collectively, these data illustrate that cells within the murine aorta express well-defined hematopoietic markers but have other phenotypic characteristics that are distinctive from BM and blood. In the context of the vessel wall’s rich CFU-M potential, Sca-1 is of particular interest due to its upregulation in ApoE-/- mice and its co-expression with Ly-6C and CD115.

Sca-1 is localized in the adventitia and enriches for CFU capacity
The distribution of Sca-1 within the vascular wall was studied by confocal microscopy. In keeping with previous reports \(^\text{14,15}\), Sca-1\(^+\) cells were most prevalent within the adventitia, in close proximity to the external elastic lamina (Figure 3A). Dual staining showed the existence of adventitial Sca-1\(^+\)c-kit\(^+\) (Figure 3B) and Sca-1\(^+\)CD45\(^+\) cells (Figure 3C) in both mouse strains and Sca-1\(^+\)CD115\(^+\) cells in \(ApoE^{\text{/-}}\) mice (Figure 3D). Sca-1 was also expressed by cells lining the vasa vasorum and to a lesser extent the intima, but not the media.

Aortas were stripped under microscopic guidance to investigate whether their CFU capacity was differentially contained within the adventitia (external layer) or media and intima (internal layer). Although immunostaining confirmed successful separation of the majority of adventitia, there remained sparse Sca-1\(^+\) adventitial cells adjacent to the external elastic lamina of internal vessel specimens. In both C57BL/6 and \(ApoE^{\text{/-}}\) mice, CFU-M yield was five-fold higher from external aortic cultures (Figure 3E). Although low frequencies of CFU-M were grown from internal layer cultures, multipotent colonies arose exclusively from the external aortic wall. Given the prevalence of Sca-1 within the CFU-rich adventitia, Sca-1\(^+\) cells were tested for their clonal potential after magnetic isolation. Colony formation, including CFU-M, was greatly enriched from the Sca-1\(^+\) fraction of aortic disaggregates, with a 50-fold difference in total yield compared to Sca-1\(^-\) cultures (Figure 3F). Therefore the hematopoietic potential of aortic cells is predominantly encompassed within the adventitial Sca-1\(^+\) population, although the innermost adventitia may be quite specific in its content of CFU-M precursors.

**Aortic cells generate hematopoietic chimerism in vivo**
To establish whether aortic cells are capable of contributing to hematopoiesis in vivo, adoptive transfer experiments were performed using aortic disaggregates from ROSA26 and GFP transgenic mice into irradiated C57BL/6 recipients. Stable chimeras were observed in peripheral blood, BM and spleen in sublethally irradiated (900cGy) mice four months after primary and secondary transplantation (Supplemental Figure 4).

To determine the frequency of repopulating cells, quantitative competitive repopulation unit (CRU) assays were conducted by transplanting limiting dilutions of aortic cells from GFP donors together with 2x10^5 C57BL/6 BM cells, into lethally irradiated (1100cGy) C57BL/6 recipients. Distinct populations of GFP^+ cells were detectable in blood and contributed to low levels of chimerism (<1-4%) four weeks after transfer. Chimeric frequency decreased over time, before plateauing somewhat in the final month of follow-up (Figure 4A; Supplemental Table 2).

Overall, the frequency of repopulating units was estimated at 1 per 4,000,000±1,300,000 aortic cells (L-calc software, Stem Cell Technologies, Inc.). At sixteen weeks, all surviving recipients of the highest cell dose (10x10^6) maintained >0.1% chimerism in blood for each lineage subpopulation (Gr1^+, CD11b^+, CD5^+, CD19^+) (Figure 4B). Donor-derived cells also commonly expressed Ly-6C, often in combination with Sca-1 (Supplemental Figure 5A). Most GFP^+ cells back-scattered to lymphocytic or monocytic morphology, while the proportion of granulocytes diminished over time (Figure 4C). Higher reconstitution was noted across all blood lineages in two mice that received 2.5x10^6 aortic cells, with one of these recipients displaying chimerism ≥1.0% over the study duration (Figure 4C, D).
Long-term presence of GFP+ cells was detected in BM, lymph nodes, spleen and liver, along with the adventitia of aorta, carotid and femoral arteries (Supplemental Figure 5B,C). In spleen, they expressed high levels of Sca-1 and markers of lymphocytic and monocytic lineage (Figure 4E; Supplemental Figure 5D). Although donor cells were strongly positive for Sca-1 and CD34 in BM, they did not display the KLS+ phenotype (Figure 4F; Supplemental Figure 5E). In this context, an important finding was that GFP+ CFUs could be cultured from recipient splenic and BM cells up to six months after transplantation, but they were exclusively CFU-M (Figure 4G). This confirmed that engrafted GFP+ cells were viable and that their fluorescent detection was not simply a by-product of phagocytic ingestion.

Taken together, infusion of aortic cells conferred a modest degree of early, multilineage hematopoietic reconstitution, with subsequent diminution to detectable, but low levels of chimerism in the long-term after primary and secondary transplantation. Although sustained clonogenicity of donor cells was observed, this was selectively restricted to macrophage colonies.

**Aortic CFUs have mixed origins in irradiated mice**

To identify the source of vascular clonal capacity, BM cells (5x10^6) from GFP donors were transplanted into lethally irradiated C57BL/6 and chow-fed ApoE−/− mice and tracked over time, focusing on their contribution to hematopoietic cells in the aorta. In non-irradiated mice, GFP+ cells were virtually absent two weeks after transfer (<0.05% in aorta, BM and blood, n=3). In irradiated recipients, GFP+ cells reconstituted BM and blood within two weeks (Figure 5A). Engraftment of donor cells occurred more gradually throughout the adventitia of blood vessels,
and to a greater extent in \textit{ApoE}^{-} mice than in C57BL/6 mice (Figure 5B-I). Although GFP\(^+\) cells reconstituted each of the Lineage (LIN\(^+\)), Sca-1\(^+\), c-kit\(^+\), CD34\(^+\) and KLS\(^+\) subpopulations in BM (Figure 5D), chimerism in aorta was strongest for LIN\(^+\) and Sca-1\(^+\) cells (Figure 5E,F). By six months, GFP\(^+\) cells isolated from recipient aorta displayed phenotypic characteristics that overlapped between cells in peripheral blood and endogenous GFP\(^-\) cells in aorta (Supplemental Figure 6).

Whole body irradiation severely reduced the CFU yield of aortic cells, which recovered only partially over the following six months (Figure 6A). The rare colonies that could be generated two days after irradiation were exclusively CFU-M of host cell origin (Figure 6B,C), indicating that donor CFU precursors were essentially absent from the aortic wall soon after transfer. In keeping with the progressive engraftment of GFP\(^+\) cells in the aorta after BM reconstitution (Figure 5), the proportion of GFP\(^+\) CFUs cultured from aortic cells also increased steadily and peaked at 43\% of total yield 90 days after transplant in C57BL/6 mice (Figure 6A). Although GFP\(^+\) colonies comprised all subtypes, they were predominantly not CFU-M, which was in direct contrast to GFP\(^-\) CFUs (Figure 6C-L). As a result, aortas displayed a mixed colony profile at 90 days, with the majority of CFU-M derived from host cells and the remaining colony types originating mostly from donor BM cells (Figure 6B-D). Over longer follow-up, CFU-M yield increased from both GFP\(^-\) and GFP\(^+\) sources, restoring the overall preponderance of macrophage colonies by six months (Figure 6B,C).

These findings demonstrate the radiation-sensitive nature of vascular hematopoietic cells. Incomplete repair of aortic CFU potential was mediated both by recovery of endogenous
macrophage precursors and progressive recruitment of BM-derived progenitors. The adventitial environment may influence the lineage commitment of trafficking myelopoietic cells by conferring specificity for monocyte/macrophage progeny over time. Further to this, we have also found that hematopoietic CFUs can be cultured from murine aortas at weaning age (3 week-old), with higher frequency than from older mice, indicating that they are present in the vascular wall from at least early postnatal life (Supplemental Figure 7).

**Discussion**

Adult blood vessels are dynamic in nature and act as reservoirs of diverse progenitor populations for smooth muscle, endothelial and mesenchymal cells. Given the intimate relationship between the vascular and hematopoietic systems during early embryonic life, we hypothesized that the postnatal vasculature may also harbor progenitor cells of hematopoietic progeny that are known to play pivotal roles in regulating vascular health and disease. Our results demonstrate that the mature murine aorta contains hematopoietic progenitors capable of self-renewal and multilineage differentiation that are primarily localized in the adventitia, under the broad immunophenotypic fingerprint of Sca-1. Although long-term hematopoietic reconstitution is restricted to a rare subpopulation of aortic cells (1 per 4,000,000 cells), the vascular wall is selectively enriched with CFU-M precursors, which are also distributed heterogeneously in other arterial territories and are upregulated in ApoE-deficient mice. Constitutive or long-standing residence, rather than recent circulatory trafficking, accounts for the majority of these cells in the aorta that may ultimately represent a local source of vascular monocytes and macrophages.
While the frequency of CFU-GEMM and GM derived from aortic disaggregates was much lower than that of BM, the aorta displayed a CFU-M-rich potential that was especially distinct from peripheral blood. This was one of several findings to verify that its hematopoietic profile as defined in this study was not due to its residual contamination with intravascular blood cells. By extension of our in vitro results, it was not surprising that in the presence of competing BM HSCs, in vivo transplantation of aortic cells resulted in low-level multilineage reconstitution of blood. Both primary and secondary transplant experiments provided evidence for long-term clonogenic recovery and self-renewal from donor aortic cells. Although the existence of multipotent HSCs, as assessed by CRU frequency, was found to be a rare event, sustained hematopoietic repopulation was impressively skewed toward monocytes and lymphocytes. Higher rates of hematopoietic reconstitution have previously been reported for fresh Sca-1+CD45+ and cultured skeletal muscle cells 8, 20 and CD45+ side population hepatic cells 9, using younger donor mice than in the present study. As distinct from the congenic CD45.1/CD45.2 model of transplantation utilized in those studies, GFP transgenic (and ROSA26) donor mice were used here to track chimerism longitudinally in C57BL/6 blood and to determine the origin of CFUs recovered from recipient BM and spleen. This approach may have under-quantified chimerism both as a result of incomplete penetrance of GFP expression in donor aortic cells, which occasionally was observed to be as low as 80%, and attenuation of GFP signal over time. The restorative hematopoietic properties of aortic disaggregates may also have been compromised by the lengthy process of enzymatic digestion, which was not required for BM cell preparation and was more intense than that used in other studies 8, 9.
Our adoptive transfer results share similarities with the levels of engraftment achieved in a prior study, in which segments of thoracic aorta were surgically transplanted under the renal capsule of irradiated mice. However, in that report long-term chimeric detection was limited to CD3+ lymphocytes and hematopoietic capacity was indirectly attributed to the proliferation of cells within the vascular intima. In contrast, our strategy of injecting aortic cells systemically was designed to facilitate their circulation to irradiated tissues, such as BM and spleen. Our in vitro findings narrow the majority of aortic CFU precursors to the adventitia and more specifically to its rich population of Sca-1+ cells. Previously, the adventitia has been described as a "vasculogenic zone", due to its high content of Sca-1+ and CD34+ vascular progenitor cells. Vascular Sca-1+ cells have already been shown to generate smooth muscle and endothelial progeny but were reported not to co-express leukocyte markers (e.g. CD45, LIN) nor form hematopoietic colonies in methylcellulose. By performing extensive flow cytometry analysis, the current study has revealed considerable immunophenotypic heterogeneity of Sca-1+ cells in the aortic wall that has not been previously acknowledged. Although Sca-1 is frequently present on cells that express c-kit or CD34, the majority of Sca-1+ cells are negative for these progenitor markers. Indeed we have found that approximately 40% of Sca-1 does co-localize with CD45, in combination with different myeloid or lymphoid-specific antigens. As mentioned above, it is the Sca-1+CD45+ phenotype that enriches for hematopoietic capacity in murine liver and skeletal muscle, notably in the absence of c-kit. c-Kit has recently been shown to identify non-hematopoietic progenitors (c-kit+VEGFR2+CD45+) in the arterial wall and this might explain the fact that despite a higher content of KLS+ cells than BM, the murine aorta contains a much lower frequency of bona fide HSCs.
Beyond its content of diverse hematopoietic and non-hematopoietic progenitors, a key finding from this study is that the aorta especially harbors lineage-directed monocyte and macrophage precursors. Monocytes play essential regulatory roles throughout the vascular system, contributing dendritic cells and macrophages/foam cells to atherosclerotic plaque and participating centrally in the early stages of angiogenesis, especially through paracrine actions. These cells exhibit phenotypic heterogeneity, characterized in mice by pro-inflammatory Ly-6CHi (CCR2+/CX3CR1Lo) and anti-inflammatory Ly-6CLo (CCR2-/CX3CR1Hi) subsets that may have both adaptive and maladaptive implications for vascular disease. Existing paradigms of monocyte/macrophage biology in cardiovascular disease have emphasized the recruitment of circulating cells from their origins in BM or spleen. However, evidence also points to the accumulation of local resident macrophages, particularly in the setting of angiogenesis, during tissue ischemia or tumor growth. To date, the precise origins of these cells have been difficult to define. While we cannot exclude that some small CFU-M may have arisen from differentiated monocytes or macrophages already established in the arterial wall, the generation of large macrophage colonies (>500 cells) and recovery of aorta-derived CFU-M from spleen and BM after primary and secondary transplantation point to the existence of highly proliferative, self-renewing precursor cells. Moreover, our results indicate that CFU-M precursors are encompassed specifically within the vascular adventitial Sca-1+ population. Further evidence indirectly linking Sca-1 to the enrichment of macrophage precursors in the aorta can be drawn from its frequent expression on Ly-6C+ cells and the finding that some adventitial Sca-1+ cells also express CD115 (c-fms or csf-1r) in the ApoE-/- strain. The c-fms receptor is the target for M-CSF on HSCs and myeloid progenitors and is implicated in the regulation of monocyte biology in inflammatory and atherosclerotic disease. These novel
immunophenotypic observations clearly distinguish the aorta from BM and blood and prompt a hierarchical view of vascular hematopoiesis that diverges from BM, where Sca-1 is lost by progenitor cells committed to the myelomonocytic lineage \(^{21}\).

Despite their low frequency in peripheral blood, HSCs are known to traffic from BM to extramedullary sites \(^{10}\) and this has been accredited as their likeliest source in skeletal muscle and liver \(^{8,9}\). Previously, it was suggested that adventitial Sca-1\(^{+}\) smooth muscle progenitors are not BM-derived, but rather constitutively maintained from early prenatal life under the imprint of sonic hedgehog signaling \(^{14,15}\). Recently, BM cell transfer was not observed to cause vascular wall engraftment in irradiated mice prior to introduction of arterial injury \(^{34}\). Focusing specifically on their contribution to vascular myelopoiesis, we found that donor-derived BM cells increasingly engrafted within the adventitia of blood vessels after BM reconstitution and contributed to its Sca-1\(^{+}\) population. Although murine adventitia contains a paucity of vasa vasorum compared to larger mammalian species, the density of these microvessels is known to be augmented in pro-atherogenic mice \(^{35}\). This provides a greater interface with the peripheral circulation for bidirectional trafficking of cells between vessel wall and remote niches (e.g. BM) and may account for the higher engraftment of GFP\(^{+}\) cells that we noted in the adventitia of \(ApoE^{-/-}\) mice compared to the C57BL/6 strain.

Allowing for the fact that the irradiation model used here caused profound hematopoietic compromise of the aorta, there were nevertheless important revelations about the nature of cellular exchange between peripheral circulation and adventitia and the likely origins of vascular myelopoietic precursors. Over time, GFP\(^{+}\) donor cells exhibited antigenic profiles in the vessel
wall that overlapped between blood (low expression of CD34, c-kit, CD150) and endogenous aorta (Sca-1^+Ly-6C^+). This suggests preferential trafficking of select cell populations to the adventitia or their phenotypic modification once engrafted there. As a reflection of this, the clonogenic properties of chimeric cells in aorta also changed from multipotent and granulocytic colonies at early time-points toward CFU-M predominance by six month follow-up. Although the setting of irradiation injury makes it difficult to draw definitive conclusions, it seems that multipotent HPCs/HSCs are most likely to arise in the aortic wall from the systemic circulation, consistent with previous findings for other postnatal tissues^8-10. Conversely, a substantial proportion of vascular CFU-M precursors appear to be either constitutively resident or the product of long-term exposure of BM-derived HPCs to the local adventitial milieu.

Pending further evaluation, the potential significance of an adventitial source of Sca-1^+ HPCs and pre-monocytes is considerable, both for the maintenance of normal vascular health and the pathogenesis of diseases, such as atherosclerosis and malignancy. The existence of these resident cells may contribute to the presence of robust leukocyte populations within the adventitia of normal and lesion-prone arteries^23, as well as the accumulation of adventitial and perivascular inflammatory cells and the expansion of vasa vasorum during the development of atherosclerosis and aneurysms^35,36. Future studies are proposed to consider whether such precursor cells might provide direct progeny to intimal atheroma in both animal models and human disease, via an "outside-in" pathway of migration, which could have highly novel implications for therapy.

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Conflict of Interest Disclosures: None.

References


**Table 1.** Immunophenotypes of single cell suspensions from C57BL/6 mice

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Aorta (%)</th>
<th>Blood (%)</th>
<th>Bone Marrow (%)</th>
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<tbody>
<tr>
<td>LIN⁺</td>
<td>24.3±2.1 (12.1-39.8)</td>
<td>92.2±2.7 § (87.1-96.1)</td>
<td>89.9±1.8§ (82.7-98.2)</td>
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<td>Sea-1⁺</td>
<td>30.8±1.5 (21.3-41.6)</td>
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<td>c-kit⁺</td>
<td>3.4±0.5 (0.3-7.2)</td>
<td>0.6±0.01 (0.6-0.6)</td>
<td>4.4±0.5 (1.8-6.2)</td>
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<td>8.4±1.0 (2.8-15.7)</td>
<td>0.7±0.4† (0.3-1.1)</td>
<td>1.5±0.4§ (0.1-4.7)</td>
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<td>2.1±0.6 (0.9-6.3)</td>
</tr>
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<td>KLS⁺</td>
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<td>0.02±0.01‡ (0.003-0.03)</td>
<td>0.3±0.1§ (0.1-0.9)</td>
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<td>0.2±0.03‡ (0.1-0.4)</td>
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<tr>
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<td></td>
<td>0.1±0.01† (0.03-0.1)</td>
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<td>32.3±3.3* (25.7-45.1)</td>
<td>3.4±0.4 (2.7-4.8)</td>
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<td>18.2±0.3§ (17.7-18.6)</td>
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<td>30.1±2.6‡ (24.9-33.3)</td>
<td>31.4±2.6‡ (28.8-33.9)</td>
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<tr>
<td>Ly-6C⁺</td>
<td>12.5±1.1 (8.4-19.1)</td>
<td>34.9±3.2‡ (24.7-50.8)</td>
<td>41.6±3.3§ (25.1-58.7)</td>
</tr>
<tr>
<td>Sca-1⁻Ly-6C⁺</td>
<td>7.4±0.8 (4.0-11.7)</td>
<td>4.0±0.9 (0.6-6.3)</td>
<td>2.2±0.2† (1.5-2.9)</td>
</tr>
</tbody>
</table>

Data represent mean±SEM percentage expression (above), with range (below), obtained from flow cytometry analysis of 3-18 C57BL/6 mice for each phenotype. *P<0.05 vs aorta, †P<0.01 vs aorta, ‡P<0.001 vs aorta, §P<0.0001 vs aorta. LIN: Lineage. KLS: c-kit⁻Lineage⁻Sca-1⁺. Statistical analysis: linear mixed effects model.
Figure Legends:

**Figure 1.** Generation of hematopoietic colony-forming units (CFUs) by aortic cells *in vitro*. (A) Examples of hematopoietic colonies after aortic cell culture in methylcellulose. (B) Frequency of different CFU types from C57BL/6 aortic cells, showing CFU-M predominance (n=15 mice). (C,D) Comparison of CFU-M and non-M yield from cells isolated from C57BL/6 blood (n=6), bone marrow (BM) (n=3) and aorta. (E,F) Generation of CFU-M, but not other CFUs, was increased from *ApoE*<sup>−/−</sup> aortic cells (n=6). Data represent mean±SEM. *P*<0.05, †*P*<0.01, ‡*P*<0.001, §*P*<0.0001. Statistical analysis: (A-D) Kruskal-Wallis test or linear mixed effect models, (E,F) Mann-Whitney test.

**Figure 2.** Co-expression of Sca-1 with progenitor and leukocyte markers on aortic cells. (A) Sca-1 expression on cells from C57BL/6 blood, BM and aorta and *ApoE*<sup>−/−</sup> aorta. §*P*<0.0001. Representative dot plots identifying (B) KLS<sup>+</sup>(150<sup>+</sup>CD34<sup>−</sup>) and (C) Sca-1<sup>+</sup>CD45<sup>+</sup> aortic cells. (D) Ly-6C was the most common leukocyte lineage marker expressed by Sca-1<sup>+</sup> aortic cells, as shown for C57BL/6 mice. *P*<0.05, †*P*<0.01 vs Ly-6C. (E,F) In contrast to BM and blood, a high proportion of aortic Ly-6C<sup>+</sup> cells were contained within the Sca-1<sup>+</sup> subpopulation, especially in *ApoE*<sup>−/−</sup> mice. ‡*P*<0.001, §*P*<0.0001 vs C57BL/6 aorta. (G) CD115 expression on *ApoE*<sup>−/−</sup> aortic cells was also predominantly associated with Sca-1, unlike in BM or blood. Graph data represent mean±SEM of ≥3 experiments. Statistical analysis: (A,E) linear mixed effect models for comparison between C57BL/6 tissues, unpaired *t*-test for comparison of *ApoE*<sup>−/−</sup> with C57BL/6 aorta, (D) one-way ANOVA with Bonferroni comparisons.
**Figure 3.** Enrichment of CFUs in the adventitia and the Sca-1\(^{+}\) population. (A) Confocal images of C57BL/6 aorta, showing IgG control (left) and Sca-1\(^{+}\) cells (red, right) densely distributed in the adventitia. Nuclei are stained blue with Hoechst. (B-D) Adventitial co-expression of Sca-1 with e-kit and CD45 in C57BL/6 aorta and with CD115 in ApoE\(^{-/-}\) aorta. Scale bars: 10\(\mu\)m (white), 5\(\mu\)m (yellow). L: lumen. (E) CFU-M yield was higher from cells isolated from the external aortic wall for both C57BL/6 (n=5) and ApoE\(^{-/-}\) (n=3) mice (left). Generation of non-M colonies from internal and external aortic layers for C57BL/6 mice (right). N.D.: not detectable. (F) Total CFU yield was enriched from Sca-1\(^{+}\) aortic cells and negligible from Sca-1\(^{-}\) cells (n=6). Data represent mean\(\pm\)SEM. *\(P<0.05\). Statistical analysis: (E) two-way ANOVA, (E,F) Wilcoxon matched-pairs signed rank test.

**Figure 4.** Hematopoietic reconstitution by aortic cells *in vivo*. (A) Time course of GFP\(^{+}\) chimerism in blood of C57BL/6 mice during CRU assays (n=7 for 10x10\(^6\) and 5x10\(^6\) groups, n=5 for 2.5x10\(^6\) and 1.25x10\(^6\)). For clarity of presentation, the 2.5x10\(^6\) group does not include data from the mouse depicted in (C) and (D). *\(P<0.05\) for dose and time comparisons. (B) Temporal profile of GFP\(^{+}\) chimerism for different lineage populations in blood, shown for the highest dose group. ‡\(P<0.001\) for CD5 expression over time, \(P\)-values for other phenotypes were not significant. (C) GFP expression in blood for the recipient of 2.5x10\(^6\) aortic cells with the highest chimerism. For each time-point, side-scatter (SSC) vs GFP signal is shown with green boxes encompassing the GFP\(^{+}\) population and the numbers representing percentage chimerism (left). The SSC vs forward-scatter (FSC) profiles of GFP\(^{+}\) cells shows diminution of granulocytes but relative maintenance of lymphocytes and monocytes (right). (D) Flow cytometry results of blood for the same mouse 16 weeks after transplantation, showing that GFP\(^{+}\) cells expressed
each lineage marker.  

\((E,F)\) GFP\(^{+}\) chimerism in subpopulations of cells harvested from \((E)\) spleen  
\((\dagger P<0.01 \text{ vs overall chimerism})\) and \((F)\) BM \((\ast P<0.05, \dagger P<0.01 \text{ vs KLS}^{+})\) in the highest dose group.  
\((G)\) GFP\(^{+}\) CFU-M cultured from recipient spleen \((\text{left})\) and BM \((\text{right})\). Scale bars: 100\(\mu\)m. Graph data represent mean±SEM. Statistical analysis: \((A)\) two-way ANOVA with repeated measures, \((B)\) one-way ANOVA with repeated measures for each phenotype, \((E,F)\) Kruskal-Wallis test.

**Figure 5.** Engraftment of BM-derived cells in the adventitia of irradiated vessels.  
\((A)\) Representative flow cytometry histograms (gray) showing rapid GFP\(^{+}\) reconstitution of blood and BM in a C57BL/6 mouse 14 days after lethal irradiation and GFP BM cell transfer.  
\((B)\) Examples at different time-points demonstrating delayed but progressive GFP expression in recipient aortas. Dotted lines show GFP expression in concurrent controls that had received only C57BL/6 BM cells.  
\((C)\) Time course of GFP\(^{+}\) chimerism in BM and aortas of C57BL/6 and Apo\(E^{-/-}\) mice after lethal irradiation and transfer (Tx) of GFP BM cells \((n \geq 3 \text{ per strain per time-point})\). \(\dagger P<0.001\) for inter-strain comparison of aortas, \(\ast P<0.0001\) for time comparison.  
\((D-F)\) Time course of GFP\(^{+}\) chimerism within subpopulations of cells from \((D)\) C57BL/6 BM, \((E)\) C57BL/6 aorta and \((F)\) Apo\(E^{-/-}\) aorta. \(\dagger P<0.01, \ast P<0.0001\).  
\((G)\) Confocal images with green filter showing adventitial distribution of chimeric GFP\(^{+}\) cells (arrowheads) in aortic arch (Arch), abdominal (Abdo) aorta and femoral (Fem) artery.  
\((H)\) Expression of Sca-1 (red) by GFP\(^{+}\) cells (green) in aortic adventitia at low and higher magnification of inset box (Hoescht = blue).  
\((I)\) Proximity of GFP\(^{+}\) (and Sca-1\(^{+}\)) cells to adventitial vasa vasorum (vWF = pale blue) in Apo\(E^{-/-}\) aortic arch. Scale bars: 10\(\mu\)m (white), 5\(\mu\)m (yellow).  
L: lumen. Data represent mean±SEM. Statistical analysis: two-way ANOVA.
**Figure 6.** Mixed origins of aortic CFUs after irradiation injury. (A) Total CFU yield from aortic cells of non-irradiated mice (n=8) and recipient C57BL/6 mice after irradiation and GFP BM cell transfer (Tx) (n≥4 for each time-point). Columns are divided into GFP+ and GFP- counts and percentages contributed by GFP+ CFUs are shown above. *P*<0.01 for comparison of GFP status, *P*<0.0001 for time comparison. (B) Temporal breakdown of aortic CFU yield into macrophage (CFU-M) and non-macrophage subtypes. Percentages of CFU-M are shown above columns. *P*<0.0001 for both CFU type and time. (C) Recovery of aortic CFU-M (*P*<0.001 for GFP status and time) and (D) non-M CFUs (*P*<0.01 for GFP status and time) showing that most macrophage colonies were of recipient origin and most non-macrophage colonies were from donor BM cells. (E-L) Examples of different CFU types generated by aortic cells from recipient mice: (E,F) GFP- CFU-M, (G-L) GFP+ CFUs. (E) and (G) are phase contrast images. Scale bars: 100μm. Data represent mean±SEM. Statistical analysis: two-way ANOVA.
Identification of a Monocyte-Predisposed Hierarchy of Hematopoietic Progenitor Cells in the Adventitia of Postnatal Murine Aorta


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Supplemental Materials

Identification of a monocyte-predisposed hierarchy of hematopoietic progenitor cells in the adventitia of postnatal murine aorta

Psaltis_Hematopoietic potential of the postnatal aorta

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Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, 55905.

Corresponding Author

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Supplemental Methods

Mice

Breeding pairs of C57BL/6 (C57BL/6J), ApoE⁻/⁻ (B6.129P2-ApoE^tm1Unc/J), GFP transgenic (C57BL/6-Tg(UBC-GFP)30Scha/J) and ROSA26 (B6;126S-Gt(ROSA)26Sor/J) mouse strains were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in the animal care facility at Mayo Clinic and maintained on standard chow diet, including those that were ApoE⁻/⁻. Both males and females were used between 12-24 weeks of age. All animal experiments complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md) and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Preparation of Single Cell Suspensions

For ex vivo cultures, flow cytometry and adoptive transfer experiments, single cell suspensions were prepared from aortas, arteries (carotid, femoral, renal), bone marrow (BM) or blood of C57BL/6, ApoE⁻/⁻, GFP or ROSA26 mice. Aorta harvests were performed under sterile conditions by pre-anesthetizing mice (ketamine 90 mg/kg, xylazine 10 mg/kg I.P.) and removing all fur before euthanasia (CO₂ inhalation). After making a longitudinal thoraco-abdominal incision, the abdominal organs were separated away and 20-40 mL of sterile phosphate buffered saline (PBS) was injected into the left ventricle to flush the aorta clear of blood, via exit incisions in the iliac arteries. The aorta was then dissected out intact, along its entire length from aortic valve to iliac bifurcation. Further flushing of the vessel was performed ex vivo to remove any residual blood contamination, before microscopic dissection of surrounding perivascular fat. In most experiments, the aorta was prepared with its mural layers intact, although in specific cases the external (adventitial) vessel layer was separated under microscopic guidance so that it could be investigated in isolation.
Vascular explants were minced into 2-3 mm pieces and incubated for 2 hours at 37°C in a solution of collagenase type I (3 mg/ml) and elastase (1 mg/ml) (Worthington Biomedical Corp., Lakewood, NJ) in Hank’s Balanced Salt Solution (HBSS, Sigma Aldrich, Inc., St Louis, MO). After digestion, aortic disaggregates were neutralized with Iscove’s Modified Dulbecco’s Medium (IMDM, Sigma Aldrich) supplemented with 10% fetal calf serum (FCS) and passed through a 40 μm filter, before washing twice. Cell counts were performed in acetic acid or 0.4% trypan blue solution to assess viability.

Where applicable, BM and peripheral blood cells were isolated and used as controls. Briefly, marrow shaft of mouse femurs and tibias were flushed with PBS and single cell suspensions prepared by drawing up and down, before filtering through 40 μm and then washing twice prior to use. Peripheral blood was drawn by intracardiac puncture or retro-orbital sampling into a small volume of ethylenediaminetetraacetic acid (EDTA). Red cells were lysed by mixing with ammonium chloride (1:10 v/v) (Stem Cell Technologies, Inc., Vancouver, Canada) at 4°C for 10 minutes. Blood cells were then washed twice with PBS. Splenic disaggregates were prepared by gentle fragmentation with the blunt end of a syringe followed by flushing with PBS through a 40 μm strainer. Skeletal muscle samples were obtained from murine hind limbs and after thorough washing, were minced and chemically digested as per the aortas, to give single cell suspensions.

**Short-term Hematopoietic Colony-Forming Unit Assays**

Washed cells (aorta, BM, blood, spleen, skeletal muscle) were resuspended in 300 μL IMDM/10% FCS and then mixed with 3 mL MethoCult® GF M3434 containing recombinant cytokines (StemCell Technologies, Inc.). Duplicate aliquots were transferred to 35 mm dishes which were incubated at 37°C, 5% CO₂ and 95% humidity for 14 days, before colony-forming
units (CFUs) were enumerated with gridded scoring dishes and classified as per manufacturer guidelines.

Short-term CFU studies were also performed with Sca-1 enriched aortic cells that were immunoselected by using the Anti-Sca-1-Microbead Kit (FITC) (Miltenyi Biotec, Gladbach, Germany). Briefly, cells were immunolabeled with anti-Sca-1-FITC, after which magnetic labeling of Sca-1+ cells was achieved with Anti-FITC Microbeads. Magnetic separation was done with the autoMACS™ Separator (Miltenyi Biotec) using positive selection program "posseld2" with purity of approximately 90%.

**Long-term Culture-Initiating Cell Assay**

The long-term culture medium was prepared from MyeloCult™ M5300 (StemCell Technologies, Inc.), supplemented with hydrocortisone in alpha Modification of Eagle's Medium (αMEM) to yield a final concentration of $10^{-6}$ M, as per the manufacturer's protocol. Whole BM cells were isolated as outlined above, plated in 96-well flat-bottom plates at $3 \times 10^5$ cells per well and then cultured for 3 weeks to create a stromal feeder layer of 70-80% confluence. The hematopoietic cells within the feeder layer were inactivated by 1500 cGy irradiation from a $^{137}$Cs γ-irradiation source. Aortic disaggregates were then added to the irradiated feeder layer at four different densities ($1.5 \times 10^4$, $3 \times 10^4$, $6 \times 10^4$ or $9 \times 10^4$ cells per well) in at least 12 replicates each. Cultures were incubated for 4 weeks at 33°C, 5% CO$_2$ and 95% humidity, changing medium weekly. After 4 weeks all the cells were detached and the contents of each well were cultured in MethoCult® GF M3434 in a 35 mm dish for 14 days. The colonies were counted, and the wells were scored positive ($\geq 1$ CFU) or negative (no CFU). Statistical analysis was performed using L-Calc software for limiting dilution analysis (Stem Cell Technologies, Inc.).
**Flow Cytometry**

Cell suspensions from the various tissue sources were resuspended in aliquots of ≤10^6 cells in 100 μl PBS/2%FCS. After blocking for 15 minutes at 4°C, cells were incubated for one hour with fluorochrome-conjugated, anti-mouse monoclonal antibodies to different progenitor and mature lineage markers: anti-Sca-1-APC, anti-c-kit-APC/Cy7, anti-CD150-Pacific Blue (PB), anti-CD34-PE, anti-CD143-PE, anti-CD115-PE, anti-CD45.2-PE, anti-Ly-6C-PB, anti-Ly-6G-APC/Cy7, anti-CD19-APC/Cy7, along with PerCP or FITC-conjugated antibodies against the lineage antigens (Ter119, CD5, CD45R/B220, CD11b, Gr1). Appropriate isotype matched controls were used. All antibodies were purchased from BioLegend (San Diego, CA), except for CD143 (R&D Systems, Inc., Minneapolis, MN). Samples were then washed and fixed in formalin/PBS for analysis with a LSRII Flow Cytometer System (BD Biosciences). List mode data files were analyzed using WinMDI version 2.9 software (Joseph Trotter, Scripps Research Institute, San Diego, CA). For the purpose of analysis, gating was performed based on light scatter morphology to exclude cell debris ¹ and expression thresholds were set at fluorescence intensity <1.0% for isotype controls.

**Tissue Immunostaining**

Intact aortic explants were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek USA, Inc., Torrance, CA). Five μm thick frozen sections were cut, fixed and blocked with 10% normal goat serum before incubating for 60 minutes with primary antibodies: rat anti-mouse Sca-1, 5 μg/ml (BD Pharmingen, San Jose, CA), rat anti-mouse CD45, 6.25 μg/ml (BD Pharmingen), rat anti-mouse CD115, 5 μg/ml (BioLegend), rabbit anti-c-kit, 2 μg/ml (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-human vWF, 15.5 μg/ml (Dako, Carpinteria, CA). Secondary antibodies were then applied using combinations of goat anti-rat Alexa Fluor 594, goat anti-rat Alexa Fluor 488, goat anti-rabbit Alexa fluor 647 (Molecular Probes, Eugene, OR) and goat anti-rabbit FITC (Santa Cruz) for 45 minutes.
Appropriate concentration matched IgG controls were used for each primary antibody. Nuclei were stained with Hoechst (Sigma Aldrich) and slides were cover-slipped using Prolong Gold mounting medium (Molecular Probes). For transplantation studies, GFP+ chimeric cells were assessed by fixing different tissue extracts (aorta, carotid and femoral arteries, spleen, liver, lymph nodes) in 10% formalin overnight and then embedding them in O.C.T. Microscopy was performed with a Zeiss LSM 510 laser scanning confocal microscope system (Carl Zeiss BmbH, Germany) (1.0 µm optical sections).

**Competitive Repopulation Unit (CRU) Transplant Studies**

Aortic disaggregates from male 12-16 week-old GFP donors were administered by tail vein injection in limiting dilutions (10x10^6, 5x10^6, 2.5x10^6, 1.25x10^6) to lethally irradiated C57BL/6 females (1100cGy, divided over two doses, 3 hours apart). An accompanying dose of 2x10^5 BM cells, prepared from two C57BL/6 male donors, was given to each recipient to provide early hematological support. Eight transplants were performed for each aortic cell dose. Control animals for each operative day received C57BL/6 BM cells only. Mice were maintained on sterile food and water containing Polymixin B (10^6 U/L) and Neomycin Sulphate (1000 mg/L).

Peripheral blood (200 µL) was taken every four weeks by retro-orbital sampling and prepared for flow cytometry analysis to quantify the level of GFP+ chimerism overall and in different myelomonocytic and lymphocytic subpopulations. Stringent flow cytometry thresholds were used for assessing GFP expression, so that in control recipients of C57BL/6 BM cells there were no GFP+ events (0.00% expression). Thus GFP expression ≥0.10% was considered to represent positive engraftment. End studies were performed four months after transplantation to determine long-term engraftment and CRU frequency, based on a Poisson distribution with L-Calc software. Recovery of GFP+ CFUs was also assessed from recipient BM and splenic cells,
using a Zeiss ApoTome microscope (Carl Zeiss GmbH) and image capture with AxioVision V4.3 software.

**Bone Marrow Trafficking Studies**

Focusing on the temporal profile with which BM-derived cells traffic to recipient aorta, 5x10^6 BM cells from GFP donors were administered to C57BL/6 and chow-fed \( \text{ApoE}^{-/-} \) mice, two hours after lethal dose irradiation. Mice were sacrificed after 2, 14, 42, 90 and 180 days (n≥9 for each strain at each time-point). GFP+ chimerism was determined quantitatively by flow cytometry for different subpopulations of aortic, BM and blood-derived cells and qualitatively by confocal microscopy of different tissue sections, including aorta and peripheral arteries. Aortic disaggregates from recipient mice were also cultured in Methocult to assess the yield and GFP status of their short-term CFUs.

**Statistical Analysis**

Data were first checked for normality of distribution by D'Agostino-Pearson omnibus test. Intergroup statistical comparisons were performed with parametric or non-parametric two-sample \( t \)-tests or ANOVA (with post-test comparisons), as appropriate. For comparisons between different tissue types harvested from the same mice, linear mixed effects models were used. Time-course data were analyzed by one-way or two-way ANOVA, with repeated measures where applicable. Results are expressed as mean±standard error (SEM) of multiple experiments, unless otherwise specified. In all cases, statistical significance was established at two-tailed \( P<0.05 \).
**Supplemental Table 1.** Surface immunophenotypes of single cell aortic suspensions

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<th>Immunophenotype</th>
<th>C57BL/6 (%)</th>
<th>ApoE⁻/⁻ (%)</th>
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<td>LIN⁺</td>
<td>24.3±2.1</td>
<td>27.3±3.4</td>
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<td>(12.1-39.8)</td>
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<td>Sca-1⁺</td>
<td>30.8±1.5</td>
<td>44.5±3.2 †</td>
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<td></td>
<td>(21.3-41.6)</td>
<td>(32.3-60.0)</td>
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<td>c-kit⁺</td>
<td>3.4±0.5</td>
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<td>(0.6-8.8)</td>
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<td>CD34⁺</td>
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<td>(2.8-15.7)</td>
<td>(3.0-16.6)</td>
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<td>CD150⁺</td>
<td>3.1±0.6</td>
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<td>(0.1-0.7)</td>
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<tr>
<td>Ly-6C⁺</td>
<td>12.5±1.1</td>
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<td>Sca-1⁺Ly-6C⁺</td>
<td>7.4±0.8</td>
<td>8.6±1.3</td>
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<td></td>
<td>(4.0-11.7)</td>
<td>(5.6-16.2)</td>
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</table>
Results represent mean±SEM percentage expression (above), with range (below), obtained from flow cytometry analysis of 4-18 C57BL/6 or ApoE⁻/⁻ mice for each phenotype. †P<0.01 for inter-strain comparison. Statistical analysis: Mann-Whitney or unpaired t-tests.
### Supplemental Table 2. GFP⁺ chimerism in peripheral blood during CRU studies

<table>
<thead>
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<th>Cell Dose</th>
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Percentage GFP expression in peripheral blood at different time-points after adoptive transfer of GFP aortic cells, with fixed dose C57BL/6 BM support. *Mice #3 and #8 in the 2.5x10^6 dosage group died under anesthesia during blood sampling at 8 weeks. Additional premature deaths, not shown here, occurred in each of the other groups within the first month of transplantation (n=1 for 10x10^6, n=1 for 5x10^6, n=3 for 1.25x10^6).
Supplemental Figure 1. Aortic disaggregates had minimal blood contamination. When resuspended in 1 mL phosphate buffered saline (PBS), aortic disaggregates had negligible hemoglobin content (0.7±0.3 g/L), compared to same-donor whole blood (136±4 g/L) or 1 mL resuspensions of whole blood pellets (60±1 g/L). Data are shown as mean±SEM from 3 mice. * P<0.05 by Kruskal-Wallis test.
Supplemental Figure 2. Distribution of macrophage colony prevalence in different arterial territories. (A) The frequency of CFU-M did not differ significantly between total cell disaggregates prepared from thoracic and abdominal aorta. (B) CFU yield was entirely restricted to CFU-M for cultures of carotid (Car) and femoral (Fem) artery cells, with a non-significant trend for reduced frequency compared to aorta (Ao). However, there was almost complete absence of colony formation after culture of renal (Ren) artery disaggregates. Data represent mean±SEM from ≥3 C57BL/6 mice for each artery type. *P<0.05 versus other arterial territories. Statistical analysis: (A) Wilcoxon matched-pairs signed rank test, (B) Kruskal-Wallis test.
Supplemental Figure 3. Disaggregated aortic cells express progenitor markers. (A) Single color flow cytometry histograms depicting representative Sca-1 staining on cells from C57BL/6 blood, BM and aorta and ApoE\(^{-/-}\) aorta. 5-15% of aortic cells expressed Sca-1 at >10^2 log fluorescence intensity, compared to <1% of peripheral blood and BM cells. MFI: mean fluorescence intensity. (B) CD34 and c-kit were also detected on the surface of aortic cells. Solid gray fills indicate antibody of interest, overlapped over the isotype matched control antibody (dotted line).
Supplemental Figure 4. Aortic cells achieve sustained chimerism after sublethal adoptive transfer. Aorta-derived cells (5x10^6), BM cells (5x10^6) or peripheral blood (100 μl) were transplanted from ROSA26 or GFP donor mice to C57BL/6 recipients that had received sublethal irradiation (900 cGy). The volume of blood was selected as it exceeds that contained within the entire aorta length. An additional control group received PBS (100 μl) only. Chimerism was assessed after four months. (A) DNA was prepared from the peripheral blood of primary recipients of ROSA26 donor cells and PCR amplified. Primers used to detect for the presence of the neomycin (Neo) cassette (867-bp product) were neo 1123 (Forward: 5’ TCC GCC TCA GAA GCC ATA GA 3’) and neo 1990 (Reverse: 5’ GAT AGC CGC GCT GCC T 3’). Aortic (Ao) cell transplantation resulted in detectable PCR product, unlike transfer of blood or...
PBS. (B) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining of cytospin preparations from recipient BM and spleen showing persistence of blue LacZ⁺ donor cells (arrowhead). (C) Representative flow cytometry dot plots of blood from a recipient four months after transfer of C57BL/6 BM cells (left) and GFP aortic cells (right), showing GFP expression and side-scatter. Numbers in quadrants denote percentage GFP expression. (D) Donor GFP⁺ engraftment (green) in the spleen of a primary recipient of aortic cells, shown at lower and higher magnification. Nuclei are stained blue with Hoechst. Scale bars: 10μm (white), 5μm (yellow). (E,F) Secondary transplants were also performed using BM cells (10x10⁶) from primary recipients. (E) PCR results showing residual detection of Neo in the blood of two secondary recipients following primary transplantation with ROSA26 aortic cells. (F) Occasional LacZ⁺ CFU-M were generated after culturing BM cells from these recipient mice (right, with LacZ⁻ CFU-M for comparison on left), indicating the self-renewal capacity of aortic CFU-M precursors.
Supplemental Figure 5. Immunophenotypic characteristics of chimeric cells after CRU assays.

Sixteen weeks after adoptive transfer in CRU assays, aorta-derived donor (GFP⁺) cells were identified in different recipient tissues, where they expressed monocyte and lymphocyte markers, along with moderate to high levels of Sca-1. (A) Flow cytometry results of recipient blood after transplantation of 10x10⁶ GFP aortic cells. Top row: GFP expression after giving...
C57BL/6 BM cells only (left) or in combination with GFP aortic cells (right). Bottom row: Dual color assessment of chimeric GFP+ cells showing their expression of Sca-1 and/or Ly-6C, with isotype matched IgGs shown for comparison. Far right: Summary of the expression of different surface antigens on GFP+ cells in blood. (B) Detection of GFP+ cells (arrowheads) in recipient spleen and liver by confocal microscopy. Nuclei are stained blue with Hoechst. (C) Homing of GFP+ cells to adventitia of aortic arch and descending aorta. Scale bars: 10µm. (D,E) Expression of different markers on donor-derived GFP+ cells, relevant to (D) myelomonocytic reconstitution in spleen and (E) progenitor cell or mature lineage phenotype in BM. Data are shown as mean±SEM from 7 mice in the 10x10^6 dose group.
Supplemental Figure 6. Phenotypic comparisons of BM-derived and local cells in irradiated aorta. Bone marrow-derived donor (GFP⁺) cells that had engrafted in the aorta six months after transplantation displayed some features consistent with their profile in recipient blood (negligible content of CD34⁺, c-kit⁺, CD150⁺, KLS⁺, KLS⁺CD150⁺ cells), but also comprised more Sca-1⁺ and Sca-1⁺Ly-6C⁺ cells, which was characteristic of recipient (GFP⁻) aortic cells. (A)
Representative flow cytometry dot plots for Sca-1 APC and CD34 PE (top) and Sca-1 APC and Ly-6C PB (bottom) in GFP+ cells isolated from recipient BM, blood and aorta and GFP- cells from recipient aorta. Numbers in quadrants represent percentage expression. Quadrant thresholds were set at fluorescence intensity giving <1.0% expression for isotype control antibodies. (B) Summaries of flow cytometry analysis comparing the expression of different markers in the overall population and GFP+ and GFP- subpopulations from recipient C57BL/6 and ApoE-/- aortas. Data represent mean±SEM from at least 3 mice six months after transplantation for each strain. *P<0.05 by Kruskal-Wallis test and Dunn post-test comparisons within depicted strain.
Supplemental Figure 7. Increased yield of aortic hematopoietic colonies from young mice.

Comparisons of short-term CFU formation are shown from aortic disaggregates of 3 week-old (weaning age) and 6 month-old mice for both C57BL/6 and ApoE⁻/⁻ strains. Data represent mean±SEM from at least 3 donor experiments for each strain and age group. *P<0.05.

Statistical analysis: Mann-Whitney test to compare different aged mice within each strain.
Supplemental References

