Characterization of a Resident Population of Adventitial Macrophage Progenitor Cells in Postnatal Vasculature


Rationale: Macrophages regulate blood vessel structure and function in health and disease. The origins of tissue macrophages are diverse, with evidence for local production and circulatory renewal.

Objective: We identified a vascular adventitial population containing macrophage progenitor cells and investigated their origins and fate.

Methods and Results: Single-cell disaggregates from adult C57BL/6 mice were prepared from different tissues and tested for their capacity to form hematopoietic colony-forming units. Aorta showed a unique predilection for generating macrophage colony-forming units. Aortic macrophage colony-forming unit progenitors coexpressed stem cell antigen-1 and CD45 and were adventitiously located, where they were the predominant source of proliferating cells in the aortic wall. Aortic Sca-1⁺CD45⁺ cells were transcriptionally and phenotypically distinct from neighboring cells lacking stem cell antigen-1 or CD45 and contained a proliferative (Ki67⁺) Lin⁻c-Kit⁺CD135⁺CD115⁻CX,CR1⁺Ly6C⁻CD11b⁻ subpopulation, consistent with the immunophenotypic profile of macrophage progenitors. Adoptive transfer studies revealed that Sca-1⁺CD45⁺ adventitial macrophage progenitor cells were not replenished via the circulation from bone marrow or spleen, nor was their prevalence diminished by depletion of monocytes or macrophages by liposomal clodronate treatment or genetic deficiency of macrophage colony-stimulating factor. Rather adventitial macrophage progenitor cells were upregulated in hyperlipidemic ApoE⁻/⁻ and LDL-R⁻/⁻ mice, with adventitial transfer experiments demonstrating their durable contribution to macrophage progeny particularly in the adventitia, and to a lesser extent the atheroma, of atherosclerotic carotid arteries.

Conclusions: The discovery and characterization of resident vascular adventitial macrophage progenitor cells provides new insight into adventitial biology and its participation in atherosclerosis and provokes consideration of the broader existence of local macrophage progenitors in other tissues. (Circ Res. 2014;115:364-375.)

Key Words: atherosclerosis ■ cells ■ leukocytes ■ macrophages ■ monocyte–macrophage precursor cells ■ progenitor cells

Members of the mononuclear phagocyte system (MPS), including macrophages and dendritic cells, mediate innate immunity and coordinate the regulation of inflammation. In the vasculature, cells of the MPS regulate vascular form and function in health and disease, highlighted by their role in atherosclerosis. While intimal macrophages are considered as a source of foam cells in atheroma, 1 adventitial macrophages also initiate plaque formation via stimulation of angiogenesis 2 and participate in the pathogenesis of inflammatory vasculopathies and aneurysm formation. 3 Although the existing paradigm of macrophage origins in vascular disease emphasizes recruitment of bone marrow–derived monocytes or splenic-derived monocytes via the circulation, 4 recent data suggest that in established atheroma, macrophage burden may be maintained predominantly by local proliferation rather than circulatory monocyte renewal. 5 This new observation fits within an emerging understanding that diversity of tissue macrophage subsets may be aligned with different developmental origins. 6, 7

Classically activated, inflammatory macrophages are generally considered to derive from circulating monocytes, which descend from hematopoietic stem and progenitor cells (HPCs). In adult murine BM, Lin⁻Sca-1⁻c-Kit⁻CD135⁻CD115⁻CX,CR1⁺ macrophage/dendritic cell progenitors (MDPs) have been identified as the common precursor for circulating monocytes and their macrophage and dendritic cell progeny, 6 with recent evidence placing Lin⁻c-Kit⁻CD135⁻CD115⁺Ly6C⁺CD11b⁺ common monocyte progenitors (cMoPs) as the link between MDPs and monocytes. 8

Original received December 20, 2013; revision received May 18, 2014; accepted June 6, 2014. In May 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.87 days.

From the Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN (P.J.P., A.S.P., D.B.S., C.D.C., S.J.H., T.A.W., S.D., L.S.K., C.S.M., S.P., R.G., R.D.S.); Monash Cardiovascular Research Centre, Monash University, Clayton, Victoria, Australia (P.J.P.); Department of Medicine, University of Adelaide, Adelaide, South Australia, Australia (P.J.P., S.D.); and Kansas University Medical Center, The University of Kansas, Kansas City (R.D.S.). The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.303299/-/DC1.

Correspondence to Robert D. Simari, Kansas University Medical Center, 3901 Rainbow Blvd, Kansas City, KS 66160. E-mail rsimari@kumc.edu

© 2014 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.303299
Despite this hierarchy of BM monocyte–macrophage ancestry, the origin of some tissue macrophages, including those belonging to the alternatively activated pathway, is less clearly defined. During prenatal development, F4/80high macrophages arise in different organs through a process that originates in the yolk sac before the onset of definitive hematopoiesis. Postnatally, the renewal, maintenance, and proliferation of certain tissue-resident macrophages occur locally, largely autonomous of BM hematopoiesis and circulatory monocyte recruitment. To date, this has been attributed to a remarkable capacity for mature tissue macrophages to undergo self-renewal, enabling them to proliferate indefinitely without loss of functional differentiation, through uncoupling of differentiation and cell cycle withdrawal by the inactivation of specific transcription factors. However, another explanation for the local proliferation and turnover of postnatal tissue macrophages, previously untested, is the existence of tissue-resident macrophage progenitor cells. With increasing recognition that postnatal blood vessels contain resident populations of diverse progenitor cell populations, we recently identified that murine arteries contain clonogenic cells of hematopoietic and nonhematopoietic adult C57BL/6 murine tissues. As described previously, great care was taken to avoid peripheral blood contamination of aortic extracts, and this was verified in numerous ways, including the use of flow cytometry (Online Figure I). Enzymatic digestion of full-length aorta from 12-week-old C57BL/6 mice yielded 1.5 to 2×10⁶ cells. Although all tissues tested generated CFUs to a varying degree, aortic cells were distinguished by a robust hematopoietic CFU capacity, which was notable for their distinct predisposition to generate lineage-specific macrophage colonies (CFU-M) at a mean frequency of 13.7±1.4 per 10⁵ aortic cells plated (n=11) or ≈200 to 300 per aorta (Figure 1A). In addition to fulfilling conventional morphological criteria, the MPS-enriched content of aortic CFU assays was supported by their substantial upregulation of macrophage and dendritic cell marker expression after 14 days of culture in methylcellulose, with absence of granulocytes (Figure 1B). Unlike aorta, CFUs generated from BM, peripheral blood, and spleen comprised much higher proportions of multilineage CFU-GM (granulocyte–macrophage) and GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte), along with CFU-G (granulocyte) and BFU-E (burst forming units-erythroid).

Methods

Detailed methods are provided in the Online Data Supplement. Single-cell tissue disaggregates were prepared from mice belonging to C57BL/6, ApoE⁻/⁻, LDL-R⁻/⁻, green fluorescent protein (GFP), and op/op (C3Fe a/a-Csf1op/CSF1op) strains under age and dietary conditions specified below. Hematopoietic colony-forming unit (CFU) assays, flow cytometry analysis, BM and splenic cell adoptive transfers, and tissue immunostaining were performed using techniques described previously. Magnetic-activated cell sorting was used to fractionate freshly isolated aortic disaggregates into subpopulations based on expression of stem cell antigen-1 (Sca-1 or Ly6A) and the pan-leukocyte marker, CD45. These different aortic cell subsets were compared for (1) clonogenicity and cell cycle activity; (2) transcriptional profiling by RNA microarray and quantitative reverse transcription polymerase chain reaction validation; (3) expression of surface markers associated with macrophage ancestry in BM, and (4) long-term in vivo fate after carotid artery adventitial transfer to atheroprone recipients. All 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

Comparisons were performed with parametric or nonparametric unpaired or paired t tests or ANOVA (with post-test comparisons), as appropriate. Results are expressed as mean±SEM of multiple experiments, unless otherwise specified. In all cases, statistical significance was established at 2-tailed P<0.05.

Results

Adventitial Sca-1⁺CD45⁺ Cells Contain Macrophage Progenitors

To understand the context and specificity of the hematopoietic potential of aorta, short-term CFU assays were performed on freshly isolated, single-cell preparations of various hematopoietic and nonhematopoietic adult C57BL/6 murine tissues. As described previously, great care was taken to avoid peripheral blood contamination of aortic extracts, and this was verified in numerous ways, including the use of flow cytometry (Online Figure I). Enzymatic digestion of full-length aorta from 12-week-old C57BL/6 mice yielded 1.5 to 2×10⁶ cells. Although all tissues tested generated CFUs to a varying degree, aortic cells were distinguished by a robust hematopoietic CFU capacity, which was notable for their distinct predisposition to generate lineage-specific macrophage colonies (CFU-M) at a mean frequency of 13.7±1.4 per 10⁵ aortic cells plated (n=11) or ≈200 to 300 per aorta (Figure 1A). In addition to fulfilling conventional morphological criteria, the MPS-enriched content of aortic CFU assays was supported by their substantial upregulation of macrophage and dendritic cell marker expression after 14 days of culture in methylcellulose, with absence of granulocytes (Figure 1B). Unlike aorta, CFUs generated from BM, peripheral blood, and spleen comprised much higher proportions of multilineage CFU-GM (granulocyte–macrophage) and GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte), along with CFU-G (granulocyte) and BFU-E (burst forming units-erythroid).

Previously, we established that arterial capacity for CFU formation is predominantly contained within the adventitial subpopulation of cells expressing Sca-1. As others have reported that adventitial Sca-1⁺Lineage⁻CD45⁺ cells possess smooth muscle differentiation potential but lack hematopoietic clonogenicity, we focused on the previously unstudied population of adventitial Sca-1⁺CD45⁺ cells. By flow cytometry, CD45 expression was present on 35.9±4.7% of Sca-1⁺ cells from C57BL/6 aortas (n=15; Figure 1C). Coexpression of Sca-1 and CD45 in healthy murine aorta was independently confirmed by tissue immunofluorescence and shown to be predominantly contained within the adventitia (Figure 1D, Online Figure II). Magnetic separation of the Sca-1⁺CD45⁺ subset of aortic cells resulted in 4-fold enrichment in hematopoietic CFU yield (Figure 1E, Online Figure III). These CFUs were exclusively CFU-M, which were more commonly of larger size than those from unfractionated cultures (Figure 1F). Exposure of Sca-1⁺CD45⁺ cells to either
Macrophage colony-stimulating factor (M-CSF) or interleukin-4 and GM colony-stimulating factor augmented CFU-M recovery (Online Figure IV A) and facilitated in vitro differentiation toward macrophage (CD11b+ F4/80+) and dendritic cell (CD11c+) phenotypes (Online Figure IVB), respectively.

By comparison to aortic Sca-1+CD45+ cells, hematopoietic colony formation was nearly absent from the Sca-1+CD45− fraction, consistent with previous depictions that these cells harbor smooth muscle and not hematopoietic progenitor capacity. Critically, colony formation was also 16-fold less from resident Sca-1−CD45+ cells, with 25% of these colonies

Figure 1. Enrichment of macrophage colonies within the aorta’s adventitial Sca-1+CD45+ compartment. A, Total and macrophage (M) colony-forming unit (CFU) counts from different tissues of 12-week-old C57BL/6 mice (n=8–13). B, Representative flow cytometry density plots of aortic CFU progeny after 14 days of methylcellulose culture showing absence of Gr-1 expression and abundance of CD11b+ F4/80+ (macrophage) and CD11b+ CD11c- (dendritic cell) staining. C, Representative flow cytometry plots of aortic disaggregates for stem cell antigen-1 (Sca-1) and CD45 expression with IgG isotype control staining also shown. D, Immunofluorescent detection of Sca-1-CD45+ coexpression in the adventitia of 12-week-old C57BL/6 aortic root. The low magnification image is stained with Hoechst for nuclei, with the yellow dotted line indicating the external elastic lamina and the inset box corresponding to the higher magnification images. Adv indicates adventitia. Scale bars: 20 μm (white) and 5 μm (yellow). Pie chart shows mean expression of the different Sca-1(S)/CD45(45) subsets in C57BL/6 aortic root adventitia from tissue immunostaining of 30 sections, 5 mice. E, ECFC frequency from different aortic Sca-1/CD45 fractions. Unf indicates unfractionated cells. n=4 to 8 different experiments per fraction, each performed from ≥6 C57BL/6 mice. *P<0.05, †P<0.01, ‡P<0.001 for comparison with Sca-1+CD45+ cells. Also see Online Figure III. F, Representative images of small (<100 cells) and large (>100 cells) CFU-M, with quantification of the proportion of large CFU-M from unfractionated and Sca-1+CD45+ cultures (n=7–10). G, Content of monocytes within the aortic Sca-1-CD45- and Sca-1+CD45+ fractions as assessed by flow cytometry (n=11). H, Nuclear Ki67 expression determined for different aortic cell subsets by flow cytometry. Total indicates all aortic cells. n=8 C57BL/6 mice. §P<0.0001 for comparison of Sca-1+CD45+ cells vs each other population. I, Proportion of cells in different aortic subpopulations that were in S or G2/M phases of cell cycle, assessed by flow cytometry of bromodeoxyuridine incorporation. n=8, P<0.0001. Also see Online Figure VI. For all graphical data, mean±SEM is shown, and *P<0.05, †P<0.01, ‡P<0.001, §P<0.0001.
comprising CFU-GM and –GEMM. The much lower yield of CFU-M from Sca-1−CD45+ cells was in spite of this population containing a higher frequency of Lin−CD11b+F4/80− monocytes than the Sca-1+CD45+ subset (Figure 1G). Along with the fact that CFU-M were generated at low frequency from peripheral blood (Figure 1A), this provided initial evidence that the cells responsible for arterial macrophage colonies are not monocytes, consistent with recent data showing the nonmonocyte origins of tissue-resident macrophages.9,11,12 This was supported in separate experiments in which freshly disaggregated aortic cells were subjected to magnetic sorting on the basis of their expression of a panel of lineage markers, which included the monocyte/macrophage antigen CD11b, along with CD5, CD19, CD45R, Gr-1, Ter119. CFU-M were almost exclusively retrieved from the lineage-depleted fraction, with negligible CFU-M recovery from the lineage-positive cells which included CD11b+ monocytes and macrophages (Online Figure V).

Together with augmented CFU-forming ability, the adventitial Sca-1+CD45+ compartment also contained the highest proportion of actively cycling cells in aorta, as corroborated by Ki67 staining analyzed by flow cytometry of aortic disaggregates (Figure 1H) and tissue immunostaining (Online Figure VI), and bromodeoxyuridine incorporation (Figure 1I, Online Figure VI). The mean rate of apoptosis, as determined by annexin V and 7-amino-actinomycin D (7AAD) uptake, was ≈10% for aortic Sca-1+CD45+ cells and did not differ significantly with aging (Online Figure VII). Thus, adventitial adventitia is enriched in macrophage progenitors that reside in the population of proliferative Sca-1+CD45+ cells.

Genomic and Phenotypic Characterization of Aortic Sca-1+CD45+ Cells

To further understand the innate differences among aortic cell populations according to their Sca-1 and CD45 expression, we performed, analyzed, and validated nonbiased transcriptional profiles of disaggregated aortic cells. Unsupervised clustering analysis of genome-wide expression arrays indicated that the combined presence of Sca-1 and CD45 was associated with extensive differences in the transcriptional profile of aortic cells (Figure 2A–2C, Online Table I). There were 44 genes that were upregulated in Sca-1+CD45+ cells compared with both the Sca-1+CD45− (resident leukocytes) and Sca-1−CD45− (smooth muscle progenitor) fractions, including those that encode Csf2 (granulocyte–macrophage colony-stimulating factor), Il5 (interleukin-5), Ccl1 (chemokine [C-C motif] ligand 1), Cxcl12 (chemokine [C–X–C motif] ligand 12), and Gata3. Many other genes were differentially regulated between Sca-1+CD45+ cells and each of the other 2 populations, with 879 and 552 genes upregulated by a factor of ≥2-fold.
compared with Sca-1−CD45+ and Sca-1−CD45− cells, respectively (Figure 2C). These covered a broad range of biological pathways, including hematopoiesis, inflammation, innate and acquired immunity, and extracellular matrix metabolism, all of which are central to vascular maintenance and disease (Figure 2D and 2E). Quantitative reverse transcription polymerase chain reaction validation of 12 of these gene products provided a Spearman r coefficient of 0.712 (P<0.01) with the microarray (Online Table II).

We used flow cytometry to characterize the hematopoietic surface markers of these subpopulations. Compared with the other Sca-1/CD45 subpopulations in adult C57BL/6 aorta, a higher percentage of Sca-1−CD45+ cells expressed CXCR1, CD115 (c-fms), and c-Kit (CD117), which are established markers of MDPs and cMoPs in BM7,8 (Figure 3A, Online Figure VIII). Further immunophenotypic characterization was then performed to examine for MDP-like (Lin−c-Kit+CD135+CD115+CXCR1+)7 and cMoP-like (Lin−c-Kit+CD135−CD115−CXCR1−Ly6C−CD11b−)8 surface marker profiles within the aortic Sca-1−CD45− fraction, where the panel of lineage markers used was CD31, NK1.1, CD3, CD19, CD11c, Ly6G, Ter119, and a live-dead marker. Although expression of the MDP-like markers accounted for <5 per 100000 aortic cells, the aortic Sca-1−CD45+ gate was found to contain a well-delineated subpopulation of cMoP-like cells (Figure 3B). The frequency of these Sca-1−CD45−Lin−c-Kit+CD135−CD115+CXCR1−Ly6C−CD11b− cells in C57BL/6 aorta was in the order of 15 to 30 per 100000 cells, which approximated the frequency of CFU-M from unfractionated aortic cell culture (see Figure 1A). This subpopulation contained a high proportion of Ki67+ cells (69.6±3.2%, n=9 mice), consistent with a proliferative progenitor (as distinct from quiescent stem cell) phenotype (Figure 3B).

Collectively, the above data indicate the distinctive genotypic and phenotypic profiles portended by the dual expression of Sca-1 and CD45 on aortic cells. The CFU-M–enriched

Figure 3. Immunophenotypic profiling of aortic Sca-1−CD45+ cells for macrophage ancestral markers. Flow cytometry gating strategies used on C57BL/6 aortic disaggregates first involved selecting single cells and excluding cell debris. A, Representative flow cytometry density plots show the expression of c-Kit, CXCR1, and CD115 on gated Sca-1−CD45+ cells. Accompanying graphs compare expression of these markers on different stem cell antigen-1 (Sca-1)/CD45 gates from C57BL/6 aorta, n=6 mice. §P<0.0001. B, C57BL/6 aorta contained a subset of Lin−Sca-1−CD45+ cells with the c-Kit+CXCR1−Ly6C−CD11b− phenotype recently used to identify common monocyte progenitors (cMoPs) in bone marrow.7 This subfraction contained a high percentage of proliferating Ki67+ cells (density plots are representative of n=9 mice), FSC-A indicates forward scatter-area; FSC-H, forward scatter-height; MDP, macrophage/dendritic cell progenitor; and SSC-A, side scatter-area. For other IgG isotype controls, see Online Figure VIII.
Sca-1^+CD45^+ fraction expresses immunophenotypic markers consistent with macrophage ancestry and includes a small subfraction of proliferative Lin^−c-Ki^+CD135^−CD115^+CX,CR1^+Ly6C^+CD11b^− cells that has been identified as cMoP-like.

**Maintenance of Aortic CFU-M Progenitors Is Not From Circulating Cells**

Our data suggest that a key difference between macrophage ancestors in BM and spleen and CFU-M progenitors in aorta is that the latter express Sca-1, as this marker is known to be downregulated and ultimately lost with progression of BM hematopoiesis to lineage specification.

This prompted us to investigate whether postnatal aortic Sca-1^−CD45^− cells and their CFU-M progeny are maintained by circulatory trafficking from BM or spleen. In the first instance, we noted that isolation of Sca-1^−CD45^− cells from BM and spleen yielded completely different CFU profiles that lacked the CFU-M specificity of the Sca-1^−CD45^− fraction in aorta (Online Figure IX). Then BM or splenic cells from GFP donors were transferred intravenously to lethally irradiated C57BL/6 mice. In both sets of adoptive transfer experiments, there was rapid reconstitution of blood with GFP^+ cells (85.8±2.8% at 2 weeks and 94.4±0.6% at 12 weeks; n=8). By 16 weeks, chimerism of GFP^+ cells in whole aorta was 15.9±3.8%, contributing to 42.9±6.9% of the aorta’s Sca-1^−CD45^− compartment but to few CD45^− cells (Figure 4A, Online Figure X). As consistent with our previous experience, recipient aortas demonstrated diminished CFU yield (total CFU at 16 weeks, 1.9±0.4 per

**Figure 4. Maintenance of aortic Sca-1−CD45− macrophage colony-forming unit (CFU-M) progenitors occurs independently of bone marrow (BM) hematopoietic progenitor cells (HPCs), blood monocytes, and aortic macrophages.**

**A.** Top left, Adoptive transfer of BM cells was performed from green fluorescent protein (GFP) donors to irradiated C57BL/6 recipients with aortic harvest at 16 weeks. Bottom right, Representative flow cytometry density plot of CD11b and F4/80 staining in aorta 16 weeks after BM cell transfer, with CD11b+F4/80^+ macrophages gated by red circle. Adjacent density plot depicts GFP^+ (donor) chimerism for the gated macrophage population, with mean±SEM % donor chimerism shown from n=6. **B**, C57BL/6 mice were treated for 30 days with liposomal clodronate or vehicle control (n=6 each group). Despite resulting in marked reduction of circulating Lin^−CD11b^+ monocytes (density plots), this was accompanied by a small increase in aortic CFU-M. **C** and **D**, Aortas were harvested from osteopetrotic (op/op) mice and their wild-type (WT) littermates (age, 16–21 days). Graphs compare (C) their respective frequencies of monocytes, macrophages, and Sca-1−CD45− cells (n=9 per group) and (D) their yield of total hematopoietic CFUs and different CFU subtypes (n=6 per group). Graphs summarize mean±SEM values. *P<0.05, †P<0.01, ‡P<0.001, §P<0.0001 (compared with Sca-1−CD45− fraction in [A]). BFU-E indicates burst-forming units–erythroid; CFU-G, CFU-granulocyte; FSC, forward scatter; GEMM, granulocyte, erythrocyte, monocyte, megakaryocyte; GM, granulocyte-macrophage; and SSC, side scatter.
circulating cells contributed to the aorta’s Sca-1+CD45+ population, those Sca-1+CD45+ cells responsible for CFU-M capacity were of host origin and not the result of recruitment to the vessel wall from BM- or splenic-derived cells. In contrast, 90.3% (56/62) of nonmacrophage colonies, including CFU-GM and -GEMM that were mostly generated from aortic Sca-1- cells, were of donor GFP+ source. These results indicate mixed sources of hematopoietic CFUs in aorta.

Importantly, in these experiments we also observed that aortic CD11b+F4/80hi macrophages were equally of both donor and recipient origin 16 weeks after irradiation and adoptive transfer. The contribution of a local source to aortic macrophages, as has been shown recently for resident macrophages in other organs,12 At the same time, it also provided additional evidence to dismiss the possibility that macrophages in the vessel wall were responsible for generating CFU-M, because 96.7% of these colonies were GFP+.

To further corroborate that aortic Sca-1+CD45+ aortic Sca-1+CD45+ cells were of donor GFP+ source. These results indicate mixed sources of hematopoietic CFUs in aorta. Circulatory surveillance of BM and splenic hematopoietic stem cells/HPCs is largely responsible for the aorta’s rare content of CFU-granulocyte, -GM, -GEMM, and burst-forming units-erythroid, consistent with the previous model proposed by Massberg et al.19 In contrast, Sca-1+CD45+ CFU-M progenitors, which are much more prevalent in aorta, are inefficiently reconstituted from BM and spleen, suggesting that they may be maintained locally.

In comparison with wild-type littermates, aortas from wean- ing-age M-CSF−/− mice had preserved numbers of Sca-1+CD45+ cells (Figure 4C) and generated similar frequencies of CFU-M under standard colony outgrowth conditions (ie, without addition of M-CSF; Figure 4D), despite the expected reduction in their frequency of circulating monocytes, as well as monocytes and macrophages in spleen (Online Figure XI) and aorta (Figure 4C). These results affirm that the aorta’s capacity to produce CFU-M is independent of cells that are reduced in op/op mice, namely circulating monocytes and monocytes and macrophages that are resident in the aortic wall. Op/op mice also had markedly diminished CFU yield from BM attributable to osteopetrosis, but enhanced hematopoiesis in spleen, resulting from displacement of hematopoietic stem cells and HPCs from BM (Online Figure XI). Interestingly, yield of nonmacrophage colonies, especially CFU-GM and -GEMM, was increased from op/op aortas (Figure 4D), demonstrating that extramedullary hematopoiesis impacted the frequency of multilineage HPCs in aorta but not macrophage-specific progenitors.

Aortic Sca-1+CD45+ Macrophage Progenitors Are Upregulated in Atherosclerosis

Hyperlipidemia has been shown to have a stimulatory effect on BM hematopoietic stem cells/HPCs, providing potential mechanistic insight into leukocyte upregulation in atherosclerotic vessels.21 However, recent evidence has suggested a switch in the predominant source of macrophages in ath- eroma of ApoE−/− mice from circulating monocytes in early lesion development to local macrophage proliferation in es- tablished intimal lesions.3 By comparison with age-matched C57BL/6 mice, we observed an increase in the frequency of Sca-1+CD45+ cells from disaggregates of whole aorta from 5-month-old ApoE−/− mice, especially in those fed 12 weeks of Western diet (WD; Figure 5A, Online Figure XII). The aortas of these hyperlipidemic mice also contained increased adventi- tial levels of M-CSF, as determined by quantitative ELISA (Online Figure XIIIB), along with increases in the generation of CFU-M (1.8-fold for chow diet ApoE−/− relative to C57BL/6; 2.3-fold for WD ApoE−/− relative to C57BL/6; Figure 5B), but not other CFU subtypes (data not shown). Similar results were observed for LDL-R−/− mice (Online Figure XIII).

Because the spleen has been implicated as an important reservoir from which proinflammatory monocytes are re- cruited to generate macrophages in atherosclerosis2,23 and myocardial infarction,24 we also interrogated the frequency of CFU-M progenitor cells in aortas of splenectomized mice. Neither CFU-M, Sca-1+CD45+ macrophage nor dendritic cell frequencies were found to be diminished in healthy, athero- prone or atherosclerotic aortas, 3 months after splenectomy, despite the anticipated reduction that splenectomy caused to aortic monocyte content (Figure 5A–5C). These data are consistent with those from our splenic cell adoptive transfer studies (Online Figure X) in verifying that the spleen is not a significant source of aortic macrophage progenitors via supply of circulating HPCs or monocytes.

We next set out to define the distribution of local macro- phage progenitor cells during murine atherosclerosis develop- ment. In aortas from C57BL/6 and prelesional chow diet ApoE−/− mice, Sca-1+CD45+ cells were almost exclusively contained within the adventitia. After 12 weeks of WD, ApoE−/− and LDL-R−/− aortas displayed transmural distribution of Sca-1+CD45+ cells, also involving media and lesional neo- intima (Figure 5D, Online Figures XII and XIII). Despite the
presence of Sca-1^+CD45^+ cells and macrophages in neointimal plaque, the great majority of CFU-M from WD ApoE^−/− aortas were still derived from adventitia, as determined when the adventitia was separated by microscopic dissection from the remaining vessel wall before digestion and culture (Figure 5E). Quantification of Ki67 nuclear expression in sections of atheroma-containing aortic root revealed that almost 75% of proliferating (Ki67^+) cells were located within the adventitia, the confines of which we defined as being within 5 cell layers from the external elastic lamina (Figure 5F and 5G). Of the 11.0±1.0% of adventitial cells that were Ki67^+ (Figure 5H), a majority were Sca-1^+CD45^+ (63.7±5.4% from 14 sections, 3 mice; Figure 5I, Online Movie I). In contrast, only 5.3±1.3% of cells in atheroma itself were Ki67^+ (P<0.001 versus adventitia), with 65.2±11.8% of these being Sca-1^+CD45^+. Overall, microscopic quantification of Ki67 staining for aortic root revealed that the highest proportion of actively cycling cells in WD ApoE^−/− aorta was contained within the Sca-1^+CD45^+ population, followed by Sca-1^−CD45^+ cells (Figure 5I). In keeping with the predominantly adventitial distribution of CFU-M progenitors in WD aorta, a higher percentage of adventitial Sca-1^+CD45^+ cells were proliferative than was the case for Sca-1^+CD45^+ cells in plaque (15.4±1.5% versus 1.8±0.6%; P<0.0001). By flow cytometry assessment of bromodeoxyuridine incorporation in disaggregates of whole aorta from WD ApoE^−/− mice, 19.8±1.2% of Sca-1^+CD45^+ cells were in the S or G2/M phases of cycle (n=10; Figure 5J). Taken together, these observations reveal spatial

Figure 5. Sca-1^+CD45^+ macrophage progenitor cells in atherosclerosis. Frequencies of (A) Sca-1^+CD45^+ cells determined by flow cytometry and (B) macrophage colony-forming units (CFU-M) from disaggregates of whole aorta from 5-month-old C57BL/6, chow diet (CD)– and Western diet (WD)–fed ApoE^−/− mice, 12 weeks after splenectomy or sham surgery (n≥6 per group). (C) Relative content of different leukocyte subsets in aortas of splenectomized WD ApoE^−/− mice (n=8) compared with sham counterparts (n=6). DCs indicates dendritic cells; Monos, monocytes; and Mφs, macrophages. (D) Immunostaining of WD ApoE^−/− aortic root showing at low magnification the presence of Sca-1^+ cells within the media and atheroma plaque (Pl). Yellow dotted lines indicate the external and internal elastic lamina.Inset box corresponds to the adjacent high magnification images showing an Sca-1^+CD45^+ cell within plaque. Graph depicts quantitative summary of the prevalence of stem cell antigen-1 (Sca-1) and CD45 expression in atheroma, adventitia (Adv), and the combination of both from 22 aortic root sections, 5 mice. (E) CFU-M yield from disaggregates of Adv or remaining vessel wall (non-Adv) after dissection of WD ApoE^−/− aortas (n=7). (F) Low- and high-power images show predominantly adventitial distribution of cycling Ki67^+ cells from WD ApoE^−/− aortic root, with (G) quantitative summary from n=14 sections, 3 mice. (H) Percentage frequency of Ki67^+ expression among all cells counted in atheroma and adventitia of these sections. (I) Pie charts summarize the distribution of Ki67 staining among the different Sca-1/CD45 subsets in atheroma, adventitia, and both. Also see Online Movie I. (J) Confirmation of the cycling nature of Sca-1^+CD45^+ cells in WD ApoE^−/− aorta by flow cytometry assessment of bromodeoxyuridine incorporation (n=10). For tissue immunostaining, nuclei are counterstained blue with Hoechst. Scale bars: 20 μm (white) and 5 μm (yellow). Graphs summarize mean±SEM values. *P<0.05, †P<0.01, ‡P<0.001, §P<0.0001.
diversity and heterogeneity of Sca-1^+CD45^+ cells within atherosclerotic aorta, highlighting that the majority of clonogenic and proliferative Sca-1^+CD45^+ macrophage progenitors do in fact remain in the adventitia.

Sca-1^+CD45^+ AMPCs Have Durable MPS Cell Fate

Finally, to interrogate the in vivo fate of Sca-1^+CD45^+ cells within atherosclerotic arteries, we isolated cells by Sca-1 and CD45 expression from aortas of GFP mice and injected each fraction into carotid artery adventitia of recipient 8-week-old ApoE^−/− mice (Figure 6A). WD was then administered for 16 weeks, during and after which GFP^+ donor cells were not detectable systemically (Figure 6B). At study end, donor-derived cells were identified in the injected artery of all recipients of Sca-1^+CD45^+ cells (n=7 mice; Figure 6C) to a greater extent than was the case 48 hours after their administration (Figure 6A). From all carotid artery sections examined from GFP^+Sca-1^+CD45^+ recipients, 89.4% (583/652) of GFP^+ cells were located in adventitia and 10.6% (69/652) in plaque (Figure 6D), although the latter was increased to 18.6% (69/371) in sections that actually contained atheroma. Of the cells enumerated in atheroma, 7.5±3.5% of cells were GFP^+ and 22.6±4.4% of all cells in adventitia were GFP^+ (Figure 6E). Phenotypic analysis revealed active cycling

Figure 6. Fate of Sca-1^+CD45^+ cells in hyperlipidemic arteries. A, Confocal images show initial adventitial retention of GFP-Sca-1^+CD45^+ cells 2 days after injection to left carotid artery of 8-week-old ApoE^−/− mouse. Ad indicates adventitia. B, Flow cytometry histograms indicate absence of GFP^+ chimerism in blood or bone marrow (BM) 12 weeks after adventitial delivery of GFP^+Sca-1^+CD45^+ cells. Dotted histograms correspond to GFP^− controls. C, Immunostaining for green fluorescent protein (GFP) expression in carotid artery sections 16 weeks after Western diet and delivery of matrigel only, GFP^+Sca-1^−CD45^+, GFP^+Sca-1^+CD45^−, or GFP^+Sca-1^+CD45^+ cells. In the 2 sections from GFP^+Sca-1^+CD45^+ cell transfer, higher magnification images of the inset boxes show donor-derived cells in adventitia (Adv) in No. 1 and atheroma plaque (Pl) in No. 2. c/l indicates contralateral (right) carotid artery; and L, lumen. D, Pie chart depicts the percentage distribution of GFP^+Sca-1^+CD45^+–derived cells between adventitia and atheroma in recipient carotid arteries (28 sections, 7 mice). E, Quantification of the percentage of cells in atheroma and adventitia that were donor-derived (GFP^+). More than 3500 nuclei assessed in all. *P<0.05. F, Costaining for GFP with F4/80, MOMA-2, and CD11c after delivery of GFP^+Sca-1^+CD45^+ cells, showing that these cells made a durable contribution to macrophage and dendritic cell progeny in recipient carotid adventitia. Representative IgG control staining is also shown. Nuclei are counterstained blue with Hoechst. Scale bars: 50 μm (orange), 20 μm (white), and 10 μm (yellow).
(Ki67+) of some donor-derived cells (Online Figure XIV), as well as expression of macrophage (F4/80, monocyte/macrophage antibody-2 [MOMA-2]) and dendritic cell (CD11c) markers (Figure 6F). By comparison, GFP was absent after injection of cell-free matrigel and was rarely identified after transfer of Sca-1−CD45+ or Sca-1+CD45− cells (Figure 6C). Although this in vivo model revealed the striking capacity of Sca-1+CD45+ cells to engrave after allogeneic transfer and provide long-term MPS progeny in their new environment, our findings suggest that the major contribution of these progenitors is to the maintenance of the adventitial, rather than neointimal, macrophage compartment, which is known to expand in atherosclerosis and play an important regulatory role in pathogenic processes, such as vasa vasorum formation.2

Discussion
Prevailing concepts of macrophage development recognize that there are diverse origins for different cellular subtypes of the MPS.5 In particular, growing evidence indicates that circulating monocytes, and by extension BM MDPs, are not the sole source of resident macrophages in many postnatal tissues.6,8,11,12 Although recent emphasis has focused on the capacity of mature macrophages to undergo self-renewal and local proliferation,6,13 an alternative inference of these observations is that there may exist local niches of tissue-resident, nonmedullary macrophage progenitor cells that persist through adult life. To address this, the current study used murine aorta as a template for the interrogation of tissue macrophage progenitor cells. Using hematopoietic CFU assays, along with a combination of genetic, phenotypic, and functional analyses, we discovered the existence of a distinct population of clonogenic adventitial progenitors that coexpress Sca-1 and CD45, are strongly predisposed to generate MPS cells, and lack granulocyte potential. A significant proportion of these cells are actively cycling in situ at any given time, under both healthy and atherosclerotic conditions. Consistent with recent evidence on the local origins of postnatal tissue macrophages, these AMPCs are not the result of circulatory trafficking of HPCs. Just as importantly, multiple lines of evidence suggest that their existence cannot be interpreted as the proliferation of monocytes recruited from the peripheral blood or the self-renewal and rapid turnover of resident mature macrophages. First, others have shown that proliferative monocytes and mature macrophages from wild-type mice are not capable of forming CFU-M.25 Second, although of variable size, the CFU-M generated by aortic Sca-1+CD45+ cells frequently comprise hundreds to thousands of cells and in prior adoptive transfer experiments have displayed remarkable long-lasting and self-renewing properties, months after primary and secondary transplantation.15 Third and fundamentally, in the present study, lineage-positive aortic cells, including those that expressed CD11b, showed minimal capacity to generate CFU-M unlike their Lin− counterparts, while the frequency of CFU-M was 16 times lower from Sca-1+CD45+ cells than Sca-1+CD45− cells, despite higher monocyte content in the former. Fourth, neither clodronate-treated C57BL/6 mice nor M-CSF−deficient mice displayed diminished aortic CFU-M yield. Fifth, in adoptive BM and splenic transfer experiments, recipient mice had ≈95% chimeric reconstitution of circulating monocytes with an almost equal mixture of donor- and recipient-derived macrophages in aorta, yet produced only 3.3% donor-derived aortic CFU-M. Sixth, aortic CFU-M were not diminished in splenectomized mice despite their reduced vessel wall content of monocytes and Ly6C0 monocytes. Finally, despite the presence of macrophages in atherosclerotic neointima, the CFU-M capacity of aortas from WD ApoE−/− mice was predominantly contained within the adventitia.

Unlike previous work reporting the presence of arterial Sca-1+CD45+ smooth muscle progenitor cells,17 our data draw attention to the fact that a considerable proportion of adventitial Sca-1+ cells express CD45 and it is within this highly heterogeneous fraction that macrophage clonogenicity is primarily contained. These cells are transcriptionally and functionally distinct from their neighboring CD45-negative counterparts. As is the case for proliferative, self-renewing microglia in the central nervous system (CNS),4,13 our results in the op/op mice indicate that the presence of Sca-1+CD45+ AMPCs is not dependent on M-CSF, although we have shown that this factor does facilitate their ability to generate CFU-M and differentiate to macrophages. Intriguingly, the presence of Sca-1 on the surface of vascular AMPCs directly contravenes classical hierarchical depictions of murine BM hematopoiesis, where Sca-1 is absent from lineage-committed myeloid precursor cells, including granulocyte–macrophage progenitors and MDPs.7 However, in keeping with the profiles ascribed to Sca-1+ BM MDPs and cMoPs,8 this study demonstrates that the adventitial Sca-1+CD45+ population does contain cells that express c-Kit, CX, CR1, and CD115 and moreover a small subset of cMoP-like Lin−c-Kit−CD135+CD115−CX, CR1+Ly6CHiCD11b− cells, which are highly proliferative and whose frequency approximates the yield of CFU-M from healthy aortas. Isolation of these rare cells from fresh aortic disaggregates by multicolor fluorescence-activated cell sorting to test their clonogenicity has thus far proven extremely challenging because of difficulties in preserving cell viability. However, it remains an important objective to further delineate the specific identity of the clonogenic AMPC subpopulation moving forward.

Although our adoptive transfer model may have been confounded by the effects of irradiation, it demonstrated that BM and splenic HPCs contribute minimally to the presence of CFU-M progenitors in postnatal aorta. Even with compromised clonal capacity after irradiation, 96.7% CFU-M and 50% of aortic macrophages were still host-derived 16 weeks after BM or splenic cell transfer, suggesting substantial local contribution. It is presumed that these macrophages were generated in the vessel wall after irradiation and were not already pre-existent there, because recent profiling of tissue and vascular macrophage kinetics has shown substantial turnover within 21 to 28 days.4,12 Future studies dedicated to investigating the BM independence of AMPCs and macrophage responses in healthy and diseased vasculature will require molecular fate-mapping strategies, similar to those used to investigate the origins of other tissue macrophages.8,11,12

Circulating monocytes and their BM-derived progenitor cells have long been considered the source of intimal macrophages and foam cells in atheroma,1 whereas the spleen has also been recognized as an intermediate reservoir of
monocytes. The present study reveals that in both mild and severe hyperlipidemia, aortic frequency of Sca-1+CD45+ expression and CFU-M yield progressively increase and this is not affected by surgical removal of the spleen and its accompanying reduction in vessel wall monocyte content. In lesional ApoE−/− and LDL-R−/− sections, we observed substantial proliferation along the adventitial aspect of the external elastic lamina, especially within the Sca-1+CD45+ population. Interestingly, although Sca-1+CD45 costaining breaches the adventitia in the setting of atheroma to involve the other mural layers, the progenitors responsible for CFU-M are still mostly adventitial. Taken together with the findings from our BM transplant studies, it is apparent that there is great diversity in the biological properties and origins of Sca-1+CD45+ cells in the vessel wall. We conclude that the vast majority of Sca-1+CD45+ cells in atheroma are neither AMPCs nor the result of adventitial-to-neointimal translocation of AMPC progeny, but are more likely to be leukocytes and lymphocytes that have originated from the circulation. The results of our carotid adventitial transfer model show that donor Sca-1+CD45+ cells, but not the other aortic subsets, generated macrophages and dendritic cells 16 weeks after delivery, although these were predominantly adventitial. This fits with the proliferative, clonogenic nature of Sca-1+CD45+ AMPCs, which are located to be able to contribute to the presence of MPS populations found in the adventitia of healthy arteries, as well as the rapid expansion of adventitial macrophages that occurs during the development of atherosclerosis and aneurysms, where these cells also help regulate growth of pathogenic vasa vasorum. We are currently focusing on the proangiogenic properties of Sca-1+CD45+ AMPCs, which we hypothesize may underlie one of their major pathophysiological functions during arterial wall remodeling. Although our results indicate that adventitial progenitors may contribute to only a small proportion of neointimal leukocytes, alternative model systems, such as molecular fate-mapping and arterial graft interposition, will be required before definitively excluding a greater role for AMPCs as a source of locally maintained macrophages in atheroma.

In summary, we have identified a population of Sca-1+CD45+ macrophage progenitor cells that reside in postnatal murine vascular adventitia. The definition of these resident progenitors provides a new context to consider the role of the adventitia in vascular disease, including atherosclerosis. Moreover, it provides a novel alternative paradigm to account for the local proliferation and turnover of tissue macrophages, advancing our understanding of the vessel wall as a source of tissue-resident macrophages and their progenitors more broadly.

Acknowledgments

We thank Megan Crouch, James Tarara, and Holly Lamb and staff of the Flow Cytometry Core Facility, Mayo Clinic, Rochester, MN.

Sources of Funding

P.J. Psaltis is funded by a Post-doctoral Overseas Biomedical Fellowship from the National Health and Medical Research Council of Australia. A.S. Puranik is funded by the James Nutter Family & Maria Long Family Fellowship in Cardiology at the Mayo Clinic.

Disclosures

None.

References

What Is Known?
• In some adult tissues, resident macrophages seem to be maintained locally, without contribution from circulating monocytes.
• Recent evidence suggests that this may also apply for macrophages in established atheroma.
• The adventitia of arteries is a niche for different progenitor cell populations.

What New Information Does This Article Contribute?
• The adventitia of postnatal murine aorta contains clonogenic macrophage progenitor cells that are Sca-1+CD45+.
• These adventitial macrophage progenitor cells (AMPCs) are independent of circulating monocytes and are not replenished by bone marrow or spleen-derived hematopoietic progenitor cells.
• AMPCs have increased prevalence in atherosclerotic aorta, where they remain predominantly adventitial, contributing macrophages to adventitia and to a lesser extent to atheroma.

Recent evidence suggests that some macrophages are not derived from bone marrow hematopoietic progenitor cells or circulating monocytes; instead, they are maintained locally, supposedly by self-renewal. Our study identifies that among different murine tissues, aorta has a robust and augmented capacity for forming macrophage colonies that arise from an adventitial population of proliferative Sca-1+CD45+ cells. These clonogenic cells are neither monocytes nor adventitial macrophages, nor are they reconstituted by circulating hematopoietic progenitor cells. AMPCs are therefore a distinct, local progenitor cell population, resident in the vessel wall. AMPCs are more abundant in the setting of atherosclerosis, providing macrophage progeny to the adventitia of atherosclerotic arteries, while also contributing to a smaller extent to atheroma cell burden. These results identify AMPCs as a new cellular participant in arterial remodeling, building on the important but underappreciated role of adventitia in vascular homeostasis and disease. Our findings suggest novel mechanisms of disease and potentially identify a new target for therapy.
Characterization of a Resident Population of Adventitial Macrophage Progenitor Cells in Postnatal Vasculature


Circ Res. 2014;115:364-375; originally published online June 6, 2014; doi: 10.1161/CIRCRESAHA.115.303299

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/115/3/364

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/06/06/CIRCRESAHA.115.303299.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Characterization of a resident population of adventitial macrophage progenitor cells in postnatal vasculature

Psaltis_Adventitial macrophage progenitor cells

Peter J. Psaltis¹,² MBBS PhD, Amrutesh Puranik¹ PhD, Daniel B. Spoon¹ MD, Colin D. Chue¹ MBChB, Scott J. Hoffman¹ MD, Tyra A. Witt¹, Sinny Delacroix¹ MD, Laurel S. Kleppe¹, Cheryl S. Mueske¹, Shuchong Pan¹ MD PhD, Rajiv Gulati¹ MD PhD, Robert D. Simari¹” MD.

¹Division of Cardiovascular Diseases, Mayo Clinic, 200 First Street SW, Rochester, MN 55905.
²Department of Medicine, University of Adelaide, North Terrace, Adelaide, South Australia, Australia, 5000

Corresponding Author
Robert D. Simari, M.D.
Kansas University Medical Center
3901 Rainbow Blvd, Kansas City, KS 66160,
Email: rsimari@kumc.edu.
Ph: 913 588-7201, Fax: 913 588-7235
Materials and Methods

Mice
Breeding pairs of C57BL/6 (C57BL/6J), ApoE⁻/⁻ (B6.129P2-ApoE<sup>tm1Unc</sup>/J), GFP (C57BL/6-Tg(UBC-GFP)30Scha/J), LDL⁻⁻ (B6.129S7-Ldl<sup>tm1Her</sup>/J) and op/op mice (C3Fe a/a-Csf1op/CSF1op 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 or Western diet (Teklad diet #88137 [42% caloric intake from fat], Harlan Laboratories, Madison, WI), as specified. Both males and females were used at ages specified throughout the text and figure legends. 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
Experiments were performed with freshly isolated, single cell suspensions from aorta or other tissues, as specified throughout the text. Preparation of aortic cell disaggregates was performed from various murine strains, as described previously<sup>1</sup>. Aorta harvests were performed under strictly sterile conditions by pre-anesthetizing mice (intraperitoneal [i.p.] ketamine 80 mg/kg, xylazine 5 mg/kg) and removing all fur before euthanasia (CO<sub>2</sub> inhalation). After application of topical antiseptic solution (betadine followed by ethanol, then repeated again), a longitudinal thoraco-abdominal incision was made and the abdominal organs were separated away. 40 mL of sterile heparinized 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 from the remaining vessel layers.

Vascular explants were minced into 2-3 mm pieces and incubated for up to 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. Typical cell counts from a single aorta varied depending on the age and size of the mouse but approximated 1.5-2 x<sup>10<sup>6</sup></sup> cells from a 12 week-old donor. We confirmed that there was absence of significant peripheral blood contamination of aortic cell
disaggregates in a variety of ways, including by quantification of hemoglobin content \(^1\) and flow cytometric analysis of Ter-119 staining (Online Figure IA) \(^2\).

Where applicable, single cell suspensions were also prepared from BM, peripheral blood, spleen, skeletal muscle, liver, heart and kidneys. Bone marrow shaft of mouse femurs and tibias were flushed with PBS, disaggregation performed by drawing up and down through a 27G needle, 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 either by gentle fragmentation with the blunt end of a syringe followed by flushing with PBS through a 40 μm strainer or alternatively enzymatic digestion with collagenase/elastase, depending on the specific experiment (Online Figure IB). Skeletal muscle samples were obtained from murine hindlimbs. Along with heart, liver and kidney, skeletal muscle isolates were washed repeatedly in PBS, before mechanical mincing and chemical digestion as per the aortas to give single cell suspensions.

**Hematopoietic colony-forming unit assays**

Washed cells from different murine tissues were resuspended in 300 μL IMDM with 10% FCS and then mixed with 3 mL MethoCult® GF M3434 containing recombinant cytokines (Stem Cell Technologies, Inc., Vancouver, Canada). Duplicate aliquots were transferred to 35 mm dishes which were incubated at 37°C, 5% CO\(_2\) 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 aortic cells that were immunoselected based on their expression of stem cell antigen-1 (Sca-1) or the pan-leukocyte antigen CD45, by using magnetic activated cell sorting (MACS).

In some experiments, after completion of CFU counts on day 14, single cell suspensions were retrieved from colonies for the purpose of immunofluorescent staining and flow cytometric characterization of leukocyte progeny. This was achieved by placing culture dishes at 4°C for two hours to liquefy methylcellulose, addition of cold IMDM and careful scraping to disaggregate and recover cells into solution. Following repeated washes and centrifugation, resulting cell pellets were prepared for flow cytometry, as detailed below.

**Magnetic activated cell sorting**

Freshly isolated, single cell aortic disaggregates were subjected to multi-column MACS, as per manufacturer recommendations (Miltenyi Biotec Inc, Auburn, CA), to obtain four subpopulations with differential expression of Sca-1 and CD45. For each separation experiment, aortas from at least six 12 week-old mice were pooled. Purity of the resulting four aortic cell fractions (Sca-1\(^+\)CD45\(^+\), Sca-
1^−CD45^−, Sca-1^−CD45^+, Sca-1^−CD45^−) was assessed by flow cytometry (Online Figure III), before setting up different in vitro and in vivo experimental assays. Bone marrow and splenic cells were also magnetically immunoselected into Sca-1/CD45 subpopulations using the same methodology.

In separate experiments, fresh aortic cell disaggregates from C57BL/6 mice were subjected to magnetic-based separation of Lineage positive and negative fractions using an EasySep negative selection kit (Stem Cell Technologies, Inc.). The panel of Lineage markers consisted of CD5, CD11b, CD19, CD45R, Gr1, Ter119.

**Flow cytometry**

Cell suspensions from the aorta or other tissue sources were resuspended in aliquots of ≤10^6 cells in 100 μL MACS buffer. After blocking for 15 minutes at 4°C, cells were incubated for 45 minutes with fluorochrome-conjugated, anti-mouse monoclonal antibodies to different cell surface markers that are cataloged in Online Table III. Appropriate isotype matched controls were used (Online Figure VII). Samples were then washed before acquiring data on either a 6-color Canto or 10-color LSRII Flow Cytometer System (BD Biosciences). List mode data files were analyzed using FlowJo software (Tree Star Inc, Ashland, OR). In certain experiments, APC-Cy7 was used as a dump channel to gate out the Lineage^+ and dead cells. Lineage markers used for flow cytometry were CD3, CD19, CD45R, Ter119, CD31, Ly6G, Near IR Live-Dead marker (Life Technologies). Expression thresholds were set at fluorescence intensity <1.0% for isotype controls.

**Analysis of cell cycle**

Flow cytometry was also used to assess Ki67 expression and incorporation of bromodeoxyuridine (BrdU) as complementary strategies for the quantification of active cell cycle within different aortic subpopulations (Online Figure VI). To investigate aortic cell incorporation of BrdU under basal in vivo conditions, BrdU was administered to 12 week-old C57BL/6 mice by i.p. injection at a dose of 1 mg (100 μL of 10 mg/mL solution). Aortas were harvested after 24 hours and fresh aortic cell disaggregates obtained, as above. BrdU uptake was determined by flow cytometry after staining with a FITC BrdU flow kit (BD Pharmingen™) and 7-amino-actinomycin (7-AAD) to label DNA, as per the manufacturer’s guidelines. Special attention was given to the percentage of cells occupying the S (synthesis) and/or G2/M (gap 2/mitosis) phases of cycle within different aortic Sca-1/CD45 subpopulations.

**Tissue immunofluorescent staining and confocal microscopy**

Intact tissue samples (aorta, aortic arch, aortic root) 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 either 10% normal goat or donkey serum, before incubating for 60 minutes with primary antibodies that are detailed in Online Table IV. Secondary antibodies were then
applied using combinations of goat or donkey-based antibodies conjugated to Alexa Fluor 594, Alexa Fluor 488 (or FITC) and Alexa fluor 647 (Molecular Probes, Eugene, OR) (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 coverslipped using Prolong Gold mounting medium (Molecular Probes). Where multiple primary antibodies were combined, every attempt was made to use reagents made in different host species to avoid false positive cross-reactivity during secondary incubation. For example, goat anti-mouse Sca-1 was used in conjunction with rat anti-mouse CD45 after verification that Sca-1 staining was adequately similar to that achieved with rat anti-mouse Sca-1.

For the purpose of detecting GFP\(^+\) chimerism in carotid adventitial transfer studies, carotid artery specimens were fixed overnight in 10% formalin and then embedded in O.C.T. Chimerism (cell retention) was first screened for by detecting endogenous EGFP expression (488 nm filter for excitation) against Hoechst-stained nuclei without processing slides through additional antigen retrieval or antibody incubation steps. Where co-expression of other antigens was tested together with GFP, antigen retrieval was first performed with the appropriate technique (usually heated citrate buffer), and GFP was labeled with either a rabbit polyclonal or rat monoclonal antibody (Online Table IV), depending on the other primary antibody (-ies) being used. Both of these anti-GFP antibodies were first validated as providing similar tissue-based detection of GFP.

Microscopy was performed with a Zeiss LSM 510 laser scanning confocal microscope system (Carl Zeiss BmbH, Germany) (1.0 µm optical sections).

**RNA microarray and RT-qPCR validation**

For the purpose of RNA isolation for microarray and RT-qPCR, aortas from six 12 week-old C57BL/6 mice per donor experiment were pooled to allow MACS-based separation of the Sca-1\(^+\)CD45\(^+\), Sca-1\(^+\)CD45\(^-\) and Sca-1\(^+\)CD45\(^-\) aortic cell fractions. Total RNA was isolated and purified immediately using an RNeasy\textsuperscript{®} Plus Mini Kit (Qiagen, Germany), as per manufacturer's instructions.

RNA microarray was performed to compare the transcriptional profiles of aortic Sca-1\(^+\)CD45\(^+\), Sca-1\(^+\)CD45\(^+\) and Sca-1\(^+\)CD45\(^-\) cells from three donor experiments. The quality of all total RNA samples was assessed according to manufacturer’s instructions for the Agilent 2100 Bioanalyzer (Santa Clara, CA). High-quality samples were labeled according to instructions for the Illumina Direct Hyb labeling method (San Diego, CA).

The log-2 of the gene expression data was normalized using the non-linear normalization `fastlo`\(^3\). To assess differential expression between groups of interest, the LIMMA package in R\(^4\) was utilized to implement the empirical Bayes method of Smyth\(^5\) to shrink the gene-wise sample variances towards a common
value. The false discovery rate (FDR), which is the expected proportion of false discoveries amongst the rejected hypotheses, was also calculated.

Pathway analysis by Ingenuity IPA (http://www.ingenuity.com/) was conducted to identify significantly enriched canonical pathways, functional groups or biological processes.

Selected genes that were differentially regulated between Sca-1+CD45+ and Sca-1-CD45+ cells and had an FDR<0.05 from microarray were validated using hydrolysis (Taqman) probe-based RT-qPCR (MIQE reference - http://miqe.genequantification.info/). All assays and reagents were ordered from Life Technologies (Foster City, CA) (Online Table II). Cq values were calculated using the Viia7 software for individual qPCR run. The data were imported and analyzed in the Expression Suite Software (Life Technologies) and the reported delta-delta Ct was plotted as log-fold difference.

**Macrophage and dendritic cell differentiation assays**

Freshly-isolated C57BL/6 aortic cells were fractionated into different Sca-1/CD45 subpopulations which were then seeded in collagen-coated glass chamber slides (Nalge Nunc International, Naperville, IL) at 2x10^4 cells in 200 μL in one of four different media conditions: (1) RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS (RPMI-10); (2) RPMI-10 with 20 ng/mL M-CSF (PeproTech Inc., Rocky Hill, NJ; (3) RPMI-10 with 20 ng/mL IL-4 and 20 ng/mL GM-CSF; (4) endothelial growth medium (Lonza, Walkersville, MD) supplemented with 10% FCS (EGM-10). Media was changed every three days until day 10, at which time the wells were fixed with cold methanol and permeated with 0.1% triton before immunostaining (as described above) to detect macrophage (CD115, F4/80), dendritic cell (CD11c) markers, with Hoechst nuclear counter-staining. Multiple images (≥10) were captured from different regions of each well under confocal microscopy (x40 objective, x0.7 zoom) to allow enumeration of the percentage of antibody-labeled cells.

**Adoptive transfer of bone marrow and splenic cells**

To determine the local versus systemic origins of aortic CFUs, adoptive transfer experiments were performed in which freshly isolated BM or splenic cells from 12 week-old ubiquitous GFP donors (5x10^6) were administered to 8 week-old C57BL/6 mice by tail vein injection two hours after lethal dose (1000 cGy) irradiation. Groups of 5-6 recipient mice were sacrificed 16 weeks later to pool their aortas to determine GFP+ chimerism by flow cytometry and to obtain different Sca-1/CD45 fractions by MACS. These aortic subpopulations were cultured in Methocult to assess their CFU yield and the GFP status (i.e. donor versus recipient origin) of their hematopoietic colonies using a Zeiss ApoTome microscope (Carl Zeiss GmbH) and image capture with AxioVision V4.3 software.
**Liposomal Clodronate Treatment**

In order to deplete circulating monocytes, C57BL/6 mice were injected intraperitoneally with clodronate liposomes (FormuMax Scientific Inc, Palo Alto, CA) 40mg/kg on the first day, followed by 20mg/kg every 3 days for a total of thirty days. Control mice received liposomes (vehicle) only.

**Splenectomy**

To investigate the possibility that the spleen may contribute CFU-M to arterial adventitia (via the circulation), mice were subjected to splenectomy or sham surgeries at eight weeks of age and then studied twelve weeks later for their aortic CFU potential and frequency of Sca-1^+CD45^+ aortic cells. Three groups of animals were investigated: chow-diet C57BL/6 and ApoE^-/- mice and Western diet ApoE^-/- mice (where Western diet was commenced immediately after splenectomy). Surgeries were performed under xylazine/ketamine (i.p.) anesthesia with incisions made just below the left costal margin to access the peritoneal cavity and spleen. Splenic blood vessels were ligated and minor surrounding vessels cauterized before removing the spleen and closing the wound with 7-0 Vicryl absorbable skin sutures placed in a continuous pattern in two layers. For sham experiments, the peritoneum was opened but the spleen not excised.

Twelve weeks after surgery, blood was sampled retro-orbitally to determine plasma cholesterol levels and percentages of circulating leukocytes and lymphocytes by flow cytometry. Euthanasia (CO₂ inhalation) was then performed and aortas harvested for preparation of single cell disaggregates for flow cytometry and methylcellulose-based CFU assay.

**Measurement of plasma cholesterol levels**

Twenty week-old mice, that had received either splenectomy or sham surgery at eight weeks of age, were fasted for four hours prior to collection of 200 μL of retro-orbital blood into an eppendorf tube containing 3.8% sodium citrate. After centrifugation, the upper layer of plasma (100 μL) was collected and cryopreserved at -80°C. Quantification of total plasma cholesterol was subsequently achieved using the Wako Cholesterol E enzyme colorimetric method (Wako Diagnostics, Wako Chemicals USA, Inc., Richmond, VA), as per manufacturer guidelines.

**ELISA-based quantification of aortic M-CSF**

Aortas were harvested in their entire length from aortic root to iliac arteries from 20 week-old, chow-diet fed C57BL/6 and ApoE^-/- mice and ApoE^-/- mice that had been maintained on a Western diet from 8 weeks of age. The adventitia was carefully dissected under microscopic guidance and then immediately frozen and maintained in liquid nitrogen until later use. Homogenates of adventitia were prepared by using homogenizer (Omni international, Kennesaw, GA) in PBS and complete protease inhibitor cocktail buffer (Roche Applied Science, Mannheim, Germany) and centrifuged at 800 x g for 10 minutes at 4°C to collect the supernatant. The protein concentration of each sample supernatant was
determined using a commercial kit following the manufacturer’s recommended protocol (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA). The content of M-CSF in supernatant fluid was determined by immunoassay in duplicate using a murine M-CSF ELISA kit (Quantikine ELISA, R&D Systems, Minneapolis, MN), as per manufacturer’s recommendations. M-CSF concentrations were normalized to total protein in the supernatant.

**Carotid adventitial delivery of aortic cells**

Aortic cells from twelve week-old GFP donor mice were freshly isolated and MACS separated into the four Sca-1/CD45 fractions (n=6 donor mice per experimental day). Cells were resuspended in matrigel (5x10^5 in 20 μL) and injected (27 G needle) as a “bleb” into the adventitia of the left carotid artery of recipient eight week-old *ApoE*⁻/⁻ mice. These surgeries were performed under xylazine/ketamine (i.p.) anesthesia with the left carotid artery exposed by a mid-longitudinal skin incision on the neck surface followed by blunt dissection of subcutaneous tissues and muscle. The right carotid artery was not exposed. Mice were maintained on a thermal pad (37°C) for at least ten minutes after matrigel/cell delivery to minimize liquefaction and leakage of the injectate. After apposing the muscle layer, skin was closed with 7-0 Vicryl sutures. In total there were five surgical groups (cell-free matrigel, n=2; Sca-1^+CD45^+ cells, n=7; Sca-1^+CD45^+ cells, n=7; Sca-1^+CD45^+ cells, n=6; Sca-1^+CD45^+ cells, n=7). Mice were commenced on a Western diet immediately after surgery, which was maintained until euthanasia 16 weeks later. At study completion, blood, bone marrow and splenic tissue were collected to assess for the systemic presence of donor-derived GFP^+ cells. Both carotid arteries were harvested, fixed and processed to investigate the presence, distribution and fate of GFP^+ cells.

**Statistical Analysis**

Statistical comparisons were performed with parametric or non-parametric unpaired or paired two-sample *t*-tests or ANOVA (with post-test comparisons), as appropriate. 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.
Online Figures and Tables
Online Figures I-XIV
Online Tables I-IV
Online Movie I
Online Figure I. Validation of methodology used for flow cytometry of tissue disaggregates

(A) Flow cytometry comparisons of CD45.2 (leukocyte) versus Ter119 (erythrocyte) content in aortic disaggregates and peripheral blood, as recommended by Galkina et al. \(^2\), as one means to verify that aortic cell preparations did not contain significant contamination with peripheral blood cells. 

(B) Flow cytometry density plots show side scatter (SSC) versus forward scatter (FSC) and Sca-1 versus CD45 expression (with IgG isotype controls) for splenic cell disaggregates prepared by mechanical filter-based separation and enzymatic digestion. Numbers in quadrants denote mean expression values from \(n=3\) mice analyzed over two separate experiments and show that the detection of Sca-1\(^+\)CD45\(^+\) cells was not altered significantly by enzymatic digestion.
Online Figure II. Adventitial co-localization of Sca-1 and CD45 staining
Confocal microscopy showing immunofluorescent detection of Sca-1+CD45+ cells in the adventitia of twelve week-old C57BL/6 aortic root. IgG control staining is shown top left. Top right: Hoechst staining of aortic root with yellow dotted line denoting the external elastic lamina. Arrows correspond to two cells that both stained positively for Sca-1 and CD45, as shown below. The cell indicated by the yellow arrow was also Ki67+. Adv=adventitia. Scales: 20μm (white), 5μm (yellow). Also see Fig. 1D and Online Figure VI.
Online Figure III. Magnetic activated cell sorting of C57BL/6 aortic cells
Representative flow cytometry density plots after magnetic separation of aortic cells into different Sca-1 (S)/CD45 (45) fractions. See also Fig 1E.
Online Figure IV. Growth factor regulation of Sca-1+CD45+ differentiation to MPS fate

(A) Fold comparisons for Sca-1+CD45+ CFU-M yield following the addition of M-CSF (20 ng/μL) or IL-4 and GM-CSF (20 ng/μL each) versus basal
methylcellulose conditions (n=3 independent experiments). (B) Detection of F4/80 and CD115 on aortic Sca-1+CD45+ cells cultured for 10 days under different conditions, with quantitative summary from three separate experiments. (C) Quantitative summary with representative examples of CD11c+ staining after aortic Sca-1+CD45+ cells were plated in RPMI or RPMI with IL-4 and GM-CSF for 10 days (n=3 independent experiments). EGM=endothelial growth medium. *P<0.05, §P<0.0001.
Online Figure V. CFU-M yield from C57BL/6 aortic cells separated by expression of Lineage panel of markers

As confirmation that the cells responsible for the aorta's capacity to produce CFU-M are neither monocytes nor macrophages, colony forming assays were performed on freshly isolated C57BL/6 aortic cells that had been MACS fractionated into Lineage (Lin) positive and negative populations, where the panel of Lineage markers included CD11b, along with CD5, CD19, CD45R, Gr1, Ter119. CFU-M recovery from the Lin pos fraction which included CD11b+ monocytes and macrophages was negligible, with enrichment of CFU-M from the Lin neg subset (n=14 per group). §P<0.0001.
Online Figure VI. Analysis of cell cycle in the aortic wall

(A) Pie chart showing distribution of Ki67 staining amongst the different Sca-1/CD45 subpopulations in the adventitia of C57BL/6 aortic root. Quantified from tissue immunostaining of n=3 mice. (B) Percentage Ki67 nuclear expression in
each Sca-1/CD45 subpopulation in aortic root adventitia, based on tissue immunostaining. *P<0.05 by ANOVA. (C) Tissue immunostaining of C57BL/6 aortic root. Lower magnification image is stained with Hoechst for nuclei and shows the different vessel wall layers, with yellow dotted line indicating the external elastic lamina and inset box corresponding to the higher magnification images below. Adv=adventitia. Scales: 20μm (white), 5μm (yellow). (D) Flow cytometry density plots showing nuclear Ki67 expression in the total population and Sca-1+CD45+ subpopulation of whole aorta. PI=propidium iodide. See also Fig 1H. (E) Flow cytometry density plots of BrdU (bromodeoxyuridine) versus 7AAD (7-aminoactinomycin) and their controls, in C57BL/6 whole aorta 24 hours after administration of intraperitoneal BrdU. Gates demonstrate cells in S (synthetic) and G2/M (gap2/mitosis) phases of cell cycle. (F) Representative flow cytometry density plots showing BrdU and 7AAD expression in different Sca-1/CD45 subsets of C57BL/6 aorta. See also Fig. 1I. (G) Representative Sca-1 versus CD45 expression for cells gated from S and S or G2/M phases of cell cycle, as depicted in (E). Graph shows mean±sem percentage of Sca-1+CD45+ expression in whole aorta, in S-phase gated aortic cells and in S or G2/M gated aortic cells from n=8 C57BL/6 mice assessed across multiple different experiments. §P<0.0001.
Online Figure VII

A

% Apoptosis

(7AAD$^+$Ann$^+$)

$S^+45^+$ $S^+45^-$ $S^+45^+$ $S^+45^+$ $S^+45^-$ $S^+45^+$

Young Old

B

CD45 Ann V 7AAD Sca-1

C

CD45 Ann V 7AAD Sca-1

×××20 μm
Online Figure VII. Analysis of apoptosis amongst Sca-1/CD45 subpopulations in C57BL/6 aorta

(A) Mean±sem data for apoptosis are shown for different Sca-1/CD45 subsets from disaggregates of 3w and 10m C57BL/6 aortas (n=4 for each age group), as determined by flow cytometry quantification of Annexin V and 7-aminoactinomycin (7AAD) co-staining. *P<0.05 by Kruskal-Wallis test between the different cell subpopulations for each of the two ages, with *P<0.05 by Bonferroni comparison. Examples are shown from image stream flow cytometry to illustrate detection of (B) live (7AAD^-AnnV^-) and (B) dead (AnnV^+) Sca-1^+CD45^+ cells from disaggregates of C57BL/6 aorta.
Online Figure VIII

**A**

**B**

**Online Figure VIII. IgG isotype control staining for flow cytometry**

Representative IgG isotype control staining of C57BL/6 aortic disaggregates, used to analyze flow cytometry experiments shown in Fig. 3 of the main manuscript. Expression thresholds were set at fluorescence intensity <1.0% for isotype controls.
Online Figure IX. CFU profiling of Sca-1/CD45 fractions from BM and spleen

Frequencies of CFUs from 14 day methylcellulose-based culture of different Sca-1/CD45 fractions of C57BL/6 (A) BM and (B) splenic cells. Adjacent pie charts show proportions of different CFU subtypes from the Sca-1*CD45+ fraction of each tissue type. Notably Sca-1*CD45+ cells from BM and spleen were not specifically predisposed to generate CFU-M, as they did almost exclusively from aorta. BFU-E=burst forming units-erythroid, G=granulocyte, GEMM=granulocyte, erythrocyte, monocyte, megakaryocyte, GM=granulocyte-macrophage, M=macrophage. Graphs depict the mean±sem of n≥3 experiments from each tissue type. *P<0.05, †P<0.01.
Online Figure X. Splenic cells do not maintain aortic CFU-M progenitors

Splenic cells from ubiquitous GFP donor mice were administered by tail vein injection to irradiated (1000 cGy) C57BL/6 recipients, with harvest and analysis of aortas sixteen weeks later. (A) Representative flow cytometry density plots for GFP versus side-scatter (SSC) from a C57BL/6 control aorta (left) and a recipient aorta after GFP+ splenic cell transplant (right). (B) Sca-1 versus CD45 expression is shown for the same recipient aorta as in (A), followed by GFP expression in the Sca-1+CD45+, Sca-1-CD45+ and Sca-1+CD45- gates. (C) Graphs show mean±sem percentage of GFP+ chimerism in different aortic fractions (left) and the total CFU yield from different aortic Sca-1/CD45 subpopulations (right), sixteen weeks after irradiation and GFP+ splenic cell transplant. N=3 experiments, each performed using six recipient aortas. *P<0.05 compared to other fractions. The accompanying table shows the numbers of total, macrophage (M) and non-macrophage (non-M) CFUs from different aortic Sca-1/CD45 fractions, along with the number and percentage of GFP+ colonies for each Sca-1/CD45 fraction and CFU subtype. As was the case after BM cell transfer (see Fig 4A), the vast majority of hematopoietic colonies from recipient aortas of splenic cell transfer were still CFU-M and these were
predominantly GFP negative and derived from the Sca-1⁺CD45⁺ fraction, indicating the host origins of Sca-1⁺CD45⁺ AMPCs. Conversely, non-M CFUs which were primarily CFU-GM and CFU-GEMM, were GFP positive and generated from Sca-1⁻ cells, indicating that their source were donor spleen-derived Sca-1⁻ HPCs.
Online Figure XI. Hematological characteristics of op/op mice deficient in M-CSF

Frequencies of hematopoietic colony forming-units (CFUs) from (A) BM and (B) splenic cells of op/op mice and their wild-type littermates (WT) (n≥3), showing marked attenuation of medullary hematopoiesis and expansion of extramedullary hematopoiesis due to osteopetrosis. Frequencies of monocytes and macrophages were reduced in (C) peripheral blood and (D) spleen of op/op mice compared to WT littermates (n=5-10). All mice were used at weaning age (16-21 days). *P<0.05, †P<0.01. See also Fig. 4C,D.
Online Figure XII. Sca-1⁺CD45⁺ cells in atherosclerotic ApoE⁻/⁻ aorta
(A) Plasma cholesterol levels in five month-old mice maintained on either chow (CD) or Western diet (WD) for twelve weeks after sham or splenectomy surgery. N>6 mice per group. †P<0.001, §P<0.0001. See also Fig. 5A-C. (B) ELISA-based quantification of M-CSF in the adventitia of aortas from CD C57BL/6 and ApoE⁻/⁻ mice and WD ApoE⁻/⁻ mice (n=6 per group). † P<0.01 compared to other strains. (C) Tissue immunostaining of aortic root from WD ApoE⁻/⁻ mouse. Low magnification images in top row demonstrate that Sca-1⁺ and CD45⁺ cells were distributed throughout all layers of the vessel wall. Adv=adventitia. Pl=atheroma plaque. The inset box corresponds to the higher magnification images below that show cells within the tunica media expressing both Sca-1 and CD45. Scales: 20μm (white), 5μm (yellow).
Online Figure XIII. Sca-1\(^+\)CD45\(^+\) expression and macrophage clonogenicity in LDL-\(R^-\) aortas

(A) Percentage frequency of Sca-1\(^+\)CD45\(^+\) cells in whole aorta disaggregates of C57BL/6, chow diet (CD) and Western diet (WD)-fed LDL-\(R^-\) mice, as determined by flow cytometry (n≥3). (B) Yield of different CFU subtypes from
aortas of LDL-R⁻/⁻ mice fed WD for twelve weeks and age-matched CD-fed C57BL/6 mice (n=7 for each). *P<0.05, †P<0.01. (C) Tissue immunostaining of aortic root from WD LDL-R⁻/⁻ mice. IgG isotype staining is shown on top. Below are low magnification images demonstrating the presence of Sca-1⁺ and CD45⁺ cells in each vessel wall layer. Adv=adventitia. Pl=atheroma plaque. The inset box corresponds to the higher magnification images in the bottom row that show a Sca-1⁺CD45⁺ cell within the atheroma plaque. Scales: 20μm (white), 5μm (yellow).
Online Figure XIV. Cycling of GFP⁺Sca-1⁺CD45⁻-derived cells after adventitial transfer

Tissue immunostaining of WD ApoE⁻/⁻ carotid artery sections sixteen weeks after adventitial injection of GFP⁺ Sca-1⁺CD45⁻ cells. Top row: low magnification images of IgG isotype staining and staining for GFP and Ki67 from two different recipient mice. In the second image in the top row, donor-derived GFP⁺ cells can be seen in atheroma plaque (Pl), adventitia (Adv) and surrounding peri-adventitial connective tissue. The white arrow denotes a GFP⁺ cell in plaque that is Ki67⁺, as shown at high magnification in the second row. In the third image in the top row, the carotid artery section does not contain atheroma, but GFP⁺ cells are present in the adventitia. The yellow arrow indicates one of these cells that is Ki67⁺ and is shown at higher magnification in the third row. Scales: 20μm (white), 5μm (yellow). Also see Fig. 6.
### Online Table IA.
Top 30 differentially regulated genes by microarray analysis,
between aortic Sca-1\(^*\)CD45\(^*\) and Sca-1\(^*\)CD45\(^*\) cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold difference</th>
<th>FDR</th>
<th>Gene</th>
<th>Fold difference</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptx3</td>
<td>18.5</td>
<td>2.4E-05</td>
<td>S100a8</td>
<td>38.0</td>
<td>3.0E-04</td>
</tr>
<tr>
<td>Tmem100</td>
<td>17.1</td>
<td>1.8E-05</td>
<td>S100a9</td>
<td>36.6</td>
<td>2.9E-04</td>
</tr>
<tr>
<td>Gfpt2</td>
<td>14.2</td>
<td>3.3E-05</td>
<td>Retnlg</td>
<td>22.6</td>
<td>1.0E-04</td>
</tr>
<tr>
<td>Cd248</td>
<td>11.9</td>
<td>1.3E-05</td>
<td>Il1b</td>
<td>20.4</td>
<td>2.2E-04</td>
</tr>
<tr>
<td>Tna</td>
<td>11.6</td>
<td>3.9E-05</td>
<td>Camp</td>
<td>19.3</td>
<td>1.3E-04</td>
</tr>
<tr>
<td>Has1</td>
<td>11.5</td>
<td>4.1E-05</td>
<td>Irg1</td>
<td>14.7</td>
<td>1.6E-04</td>
</tr>
<tr>
<td>Angptl1</td>
<td>10.9</td>
<td>2.5E-05</td>
<td>C1qc</td>
<td>13.2</td>
<td>6.9E-05</td>
</tr>
<tr>
<td>Gpc3</td>
<td>10.8</td>
<td>1.3E-05</td>
<td>Lyz1</td>
<td>13.0</td>
<td>2.1E-04</td>
</tr>
<tr>
<td>Scara5</td>
<td>10.4</td>
<td>1.8E-05</td>
<td>Lyz2</td>
<td>12.8</td>
<td>4.7E-03</td>
</tr>
<tr>
<td>Lgi2</td>
<td>10.3</td>
<td>2.4E-05</td>
<td>Chi3l3</td>
<td>12.5</td>
<td>3.6E-04</td>
</tr>
<tr>
<td>Lrrn4cl</td>
<td>10.3</td>
<td>1.3E-05</td>
<td>F13a1</td>
<td>12.2</td>
<td>6.9E-05</td>
</tr>
<tr>
<td>Gsn</td>
<td>9.9</td>
<td>4.5E-04</td>
<td>C1qa</td>
<td>11.8</td>
<td>1.6E-04</td>
</tr>
<tr>
<td>Pi16</td>
<td>9.4</td>
<td>2.4E-05</td>
<td>Lcn2</td>
<td>11.8</td>
<td>1.7E-04</td>
</tr>
<tr>
<td>Pla1a</td>
<td>9.2</td>
<td>2.9E-05</td>
<td>Mrc1</td>
<td>11.8</td>
<td>7.7E-05</td>
</tr>
<tr>
<td>Clec3b</td>
<td>9.0</td>
<td>1.3E-05</td>
<td>Ccl3</td>
<td>11.5</td>
<td>8.9E-05</td>
</tr>
<tr>
<td>C7</td>
<td>8.7</td>
<td>1.9E-04</td>
<td>Mpo</td>
<td>11.1</td>
<td>2.6E-03</td>
</tr>
<tr>
<td>Sfrp4</td>
<td>8.6</td>
<td>8.0E-05</td>
<td>Gpr109a</td>
<td>10.7</td>
<td>1.7E-04</td>
</tr>
<tr>
<td>Ccl11</td>
<td>8.2</td>
<td>2.4E-05</td>
<td>Ccl4</td>
<td>10.6</td>
<td>2.4E-04</td>
</tr>
<tr>
<td>Srpx</td>
<td>7.8</td>
<td>1.8E-05</td>
<td>Ccl9</td>
<td>10.2</td>
<td>6.0E-05</td>
</tr>
<tr>
<td>Avpr1a</td>
<td>7.8</td>
<td>3.0E-05</td>
<td>Myh11</td>
<td>10.2</td>
<td>8.0E-05</td>
</tr>
<tr>
<td>Serpina3n</td>
<td>7.3</td>
<td>4.1E-05</td>
<td>Ccl5</td>
<td>10.1</td>
<td>3.8E-04</td>
</tr>
<tr>
<td>Pcsk6</td>
<td>7.3</td>
<td>3.5E-05</td>
<td>Ngp</td>
<td>10.1</td>
<td>5.3E-04</td>
</tr>
<tr>
<td>Gpnmb</td>
<td>7.0</td>
<td>5.2E-05</td>
<td>Fcrls</td>
<td>10.0</td>
<td>6.4E-05</td>
</tr>
<tr>
<td>Vit</td>
<td>7.0</td>
<td>3.4E-05</td>
<td>Clec4d</td>
<td>10.0</td>
<td>6.9E-05</td>
</tr>
<tr>
<td>Xpnpep2</td>
<td>7.0</td>
<td>2.4E-05</td>
<td>Acta2</td>
<td>9.9</td>
<td>2.4E-04</td>
</tr>
<tr>
<td>Islr</td>
<td>6.9</td>
<td>3.5E-04</td>
<td>Alox5ap</td>
<td>9.8</td>
<td>4.1E-05</td>
</tr>
<tr>
<td>Spon2</td>
<td>6.9</td>
<td>1.8E-05</td>
<td>C1qa</td>
<td>9.8</td>
<td>4.1E-05</td>
</tr>
<tr>
<td>Igfbp6</td>
<td>6.8</td>
<td>4.0E-05</td>
<td>Ifnb1</td>
<td>9.3</td>
<td>6.0E-05</td>
</tr>
<tr>
<td>Bmp8</td>
<td>6.8</td>
<td>4.0E-05</td>
<td>Msr2</td>
<td>9.3</td>
<td>9.3E-05</td>
</tr>
<tr>
<td>Amy2-2</td>
<td>6.8</td>
<td>1.5E-05</td>
<td>Folr2</td>
<td>9.2</td>
<td>1.4E-04</td>
</tr>
<tr>
<td>Gene</td>
<td>Fold difference</td>
<td>FDR</td>
<td>Gene</td>
<td>Fold difference</td>
<td>FDR</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------</td>
<td>--------</td>
<td>---------</td>
<td>-----------------</td>
<td>--------</td>
</tr>
<tr>
<td>Igk-C</td>
<td>194.9</td>
<td>1.7E-04</td>
<td>Acta2</td>
<td>4.3</td>
<td>8.3E-03</td>
</tr>
<tr>
<td>Ccl4</td>
<td>92.2</td>
<td>5.5E-05</td>
<td>Myh11</td>
<td>3.9</td>
<td>3.5E-03</td>
</tr>
<tr>
<td>IgI-V1</td>
<td>91.8</td>
<td>5.5E-05</td>
<td>4cytl1</td>
<td>3.9</td>
<td>3.9E-04</td>
</tr>
<tr>
<td>Igh-6</td>
<td>84.1</td>
<td>2.0E-04</td>
<td>Enpp6</td>
<td>3.2</td>
<td>6.1E-03</td>
</tr>
<tr>
<td>Cma2</td>
<td>79.2</td>
<td>7.0E-04</td>
<td>Plp1</td>
<td>3.2</td>
<td>2.6E-03</td>
</tr>
<tr>
<td>Iggh</td>
<td>72.3</td>
<td>1.7E-03</td>
<td>Adh7</td>
<td>3.2</td>
<td>4.5E-03</td>
</tr>
<tr>
<td>Igk-V5</td>
<td>69.9</td>
<td>3.4E-05</td>
<td>Adra2a</td>
<td>3.2</td>
<td>4.2E-04</td>
</tr>
<tr>
<td>Cpa3</td>
<td>61.7</td>
<td>7.0E-04</td>
<td>Klk10</td>
<td>3.1</td>
<td>2.8E-03</td>
</tr>
<tr>
<td>Cd52</td>
<td>60.0</td>
<td>1.4E-04</td>
<td>Gja5</td>
<td>2.9</td>
<td>7.0E-04</td>
</tr>
<tr>
<td>Mcpt4</td>
<td>55.4</td>
<td>8.8E-04</td>
<td>Fblim1</td>
<td>2.8</td>
<td>1.2E-03</td>
</tr>
<tr>
<td>Laptm5</td>
<td>50.1</td>
<td>5.2E-05</td>
<td>Irf6</td>
<td>2.7</td>
<td>2.2E-03</td>
</tr>
<tr>
<td>Cd74</td>
<td>44.0</td>
<td>7.0E-04</td>
<td>Fmod</td>
<td>2.7</td>
<td>8.2E-03</td>
</tr>
<tr>
<td>Faim3</td>
<td>40.9</td>
<td>7.0E-04</td>
<td>Dhh</td>
<td>2.6</td>
<td>1.4E-03</td>
</tr>
<tr>
<td>Coro1a</td>
<td>40.5</td>
<td>1.2E-04</td>
<td>Bmp4</td>
<td>2.6</td>
<td>1.1E-03</td>
</tr>
<tr>
<td>Cma1</td>
<td>29.4</td>
<td>2.2E-03</td>
<td>Itih4</td>
<td>2.6</td>
<td>2.1E-02</td>
</tr>
<tr>
<td>Tcrb-V8.2</td>
<td>28.9</td>
<td>5.7E-04</td>
<td>Pdlim3</td>
<td>2.5</td>
<td>1.9E-02</td>
</tr>
<tr>
<td>H2-Ab1</td>
<td>27.3</td>
<td>9.2E-04</td>
<td>Myl9</td>
<td>2.5</td>
<td>3.6E-02</td>
</tr>
<tr>
<td>Cytip</td>
<td>26.1</td>
<td>7.0E-04</td>
<td>Smoc1</td>
<td>2.5</td>
<td>7.0E-04</td>
</tr>
<tr>
<td>H2-Eb1</td>
<td>25.4</td>
<td>7.0E-04</td>
<td>Mylk</td>
<td>2.5</td>
<td>6.1E-03</td>
</tr>
<tr>
<td>Mcpt6</td>
<td>22.6</td>
<td>1.3E-03</td>
<td>Actg2</td>
<td>2.4</td>
<td>2.6E-02</td>
</tr>
<tr>
<td>Cd3g</td>
<td>19.3</td>
<td>7.0E-04</td>
<td>Lamc3</td>
<td>2.4</td>
<td>2.6E-03</td>
</tr>
<tr>
<td>Cd3d</td>
<td>17.6</td>
<td>7.0E-04</td>
<td>Col15a1</td>
<td>2.4</td>
<td>6.1E-03</td>
</tr>
<tr>
<td>Cd79b</td>
<td>15.9</td>
<td>2.6E-03</td>
<td>Ncam1</td>
<td>2.4</td>
<td>3.3E-02</td>
</tr>
<tr>
<td>Sla</td>
<td>15.3</td>
<td>9.2E-04</td>
<td>Zf hx3</td>
<td>2.4</td>
<td>2.5E-03</td>
</tr>
<tr>
<td>Cd3e</td>
<td>15.1</td>
<td>7.0E-04</td>
<td>Trim2</td>
<td>2.3</td>
<td>7.0E-04</td>
</tr>
<tr>
<td>Cd8b1</td>
<td>15.0</td>
<td>6.0E-03</td>
<td>Nkx2-3</td>
<td>2.3</td>
<td>2.8E-03</td>
</tr>
<tr>
<td>Fcer1g</td>
<td>14.4</td>
<td>1.1E-03</td>
<td>Kcnmb1</td>
<td>2.3</td>
<td>1.3E-02</td>
</tr>
<tr>
<td>Nup210</td>
<td>13.8</td>
<td>1.7E-03</td>
<td>Crispld2</td>
<td>2.3</td>
<td>1.8E-03</td>
</tr>
<tr>
<td>Sell</td>
<td>13.5</td>
<td>2.8E-03</td>
<td>Dio2</td>
<td>2.3</td>
<td>1.2E-02</td>
</tr>
<tr>
<td>C1qb</td>
<td>13.5</td>
<td>7.0E-04</td>
<td>Art4</td>
<td>2.3</td>
<td>2.8E-03</td>
</tr>
</tbody>
</table>

Online Table IB. Top 30 differentially regulated genes by microarray analysis, between aortic Sca-1+CD45+ and Sca-1+CD45- cells
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Assay-on-demand reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>18s</td>
<td>Mm03928990_g1</td>
</tr>
<tr>
<td>Ace</td>
<td>Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1</td>
<td>Mm00802048_m1</td>
</tr>
<tr>
<td>Actb</td>
<td>Beta-actin</td>
<td>Mm00607939_s1</td>
</tr>
<tr>
<td>Apoe</td>
<td>Apolipoprotein E</td>
<td>Mm01307193_g1</td>
</tr>
<tr>
<td>Ccl11</td>
<td>Chemokine (C-C motif) ligand 11</td>
<td>Mm00441238_m1</td>
</tr>
<tr>
<td>Cd248</td>
<td>CD248 molecule, endosialin</td>
<td>Mm00547485_s1</td>
</tr>
<tr>
<td>Csf1</td>
<td>Colony stimulating factor 1 (macrophage)</td>
<td>Mm00432686_m1</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Mm99999915_g1</td>
</tr>
<tr>
<td>Ldlr</td>
<td>Low-density-lipoprotein receptor</td>
<td>Mm00440169_m1</td>
</tr>
<tr>
<td>Ly6a</td>
<td>Lymphocyte antigen 6 complex, locus A</td>
<td>Mm00726565_s1</td>
</tr>
<tr>
<td>Ptx3</td>
<td>Pentraxin 3, long</td>
<td>Mm00477268_m1</td>
</tr>
<tr>
<td>Vegfa</td>
<td>Vascular endothelial growth factor A</td>
<td>Mm01281449_m1</td>
</tr>
<tr>
<td>Vegfc</td>
<td>Vascular endothelial growth factor C</td>
<td>Mm00437310_m1</td>
</tr>
<tr>
<td>Vldlr</td>
<td>Very-low-density-lipoprotein receptor</td>
<td>Mm00443298_m1</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>Wingless related MMTV integration site 5a</td>
<td>Mm00437347_m1</td>
</tr>
<tr>
<td>Wnt10b</td>
<td>Wingless related MMTV integration site 10b</td>
<td>Mm00442104_m1</td>
</tr>
</tbody>
</table>

Online Table II. Genes acquired from Life Technologies and used for RT-qPCR validation of microarray data.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>BV711, FITC, PE-Cy7, PerCP</td>
<td>M1/70</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>APC-Cy7, FITC, PE</td>
<td>N418</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD115 (CSF-1R)</td>
<td>APC, PE</td>
<td>AFS98</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD117 (c-Kit)</td>
<td>BV421, PE-CF594</td>
<td>2B8</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD45.2</td>
<td>FITC, PE, PerCP</td>
<td>104</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CX3CR1 (Goat polyclonal anti-mouse)</td>
<td>PE</td>
<td></td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>F4/80</td>
<td>APC, BV421, FITC</td>
<td>BM8</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Ki-67</td>
<td>FITC, PE/Cy7</td>
<td>B56</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Ly6A/E (Sca-1)</td>
<td>APC, eF605NC</td>
<td>D7</td>
<td>Biolegend, eBioscience</td>
</tr>
<tr>
<td>Ly-6C</td>
<td>APC, Pacific blue, PE-CF594</td>
<td>HK1.4</td>
<td>Biolegend, BD Biosciences</td>
</tr>
<tr>
<td>Ly-6G/Ly-6C (Gr-1)</td>
<td>APC-Cy7, FITC</td>
<td>RB6-8C5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Ter-119, CD3, CD19, CD45R (B220), CD31, Ly6G (A18), Brdu Flow kit (7AAD, Dnase, FITC anti-BRDU)</td>
<td>APC-Cy7, FITC</td>
<td></td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

**Other**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>7AAD</td>
<td></td>
<td>eBioscience</td>
</tr>
<tr>
<td>Annexin V</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>FcR blocking reagent, mouse</td>
<td></td>
<td>Miltenyi Biotec</td>
</tr>
<tr>
<td>Near IR Live-Dead marker</td>
<td></td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td></td>
<td>BD Pharmingen</td>
</tr>
</tbody>
</table>

**Online Table III. Antibodies and reagents used for flow cytometry**
### Antibody Controls
- Armenian hamster IgG, monoclonal: Biolegend, San Diego, CA
- Goat IgG, polyclonal: Sigma, St Louis, MO
- Mouse IgG, monoclonal: R&D Systems, Minneapolis, MN
- Rabbit IgG, polyclonal: R&D Systems
- Rat IgG₂κ, monoclonal: BD Pharmingen, San Jose, CA
- Rat IgG₂βκ, monoclonal: BD Pharmingen

### Primary Antibodies
- Armenian hamster anti-mouse CD11c: Abcam, Cambridge, MA
- Goat anti-mouse Sca-1: R&D Systems
- Rat anti-GFP: MBL, Woods Hole, MA
- Rat anti-mouse CD115: Biolegend
- Rat anti-mouse CD45: BD Pharmingen
- Rat anti-mouse/human F4/80: Abcam
- Rat anti-mouse MOMA-2: Abcam
- Rat anti-mouse Sca-1: BD Pharmingen
- Rabbit anti-GFP: Life Technologies, Molecular Probes, Grand Island, NY
- Rabbit anti-Ki67: Abcam

### Secondary Antibodies
- Donkey anti-goat AF488, AF647: Life Technologies
- Donkey anti-rabbit AF647: Life Technologies
- Donkey anti-rat AF488, AF594: Life Technologies
- Donkey anti-mouse AF488: Life Technologies
- Goat anti-hamster AF488: Life Technologies
- Goat anti-mouse AF594, AF647: Life Technologies
- Goat anti-rat AF488, AF594, AF647: Life Technologies
- Goat anti-rabbit AF488, AF594, AF647: Life Technologies

### Other
- Donkey serum: Sigma
- Goat serum: Sigma
- Hoechst: Sigma

---

**Online Table IV. Antibodies and reagents used for tissue immunofluorescent staining**

AF= Alexa Fluor
Online Movie I. Z-stack reconstruction of Ki67$^+$Sca-1$^+$CD45$^+$ cell from WD ApoE$^{-/-}$ aortic root adventitia
Ki67= cyan; Sca-1= red; CD45= green; nucleus stained blue with Hoechst.
Supplemental References


