Extramedullary Hematopoiesis Generates Ly-6C<sup>high</sup> Monocytes that Infiltrate Atherosclerotic Lesions

Running title: Robbins et al.; Extramedullary hematopoiesis in atherosclerosis

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Abstract:

**Background** - Atherosclerotic lesions are believed to grow via the recruitment of bone marrow-derived monocytes. Among the known murine monocyte subsets, Ly-6C<sup>high</sup> monocytes are inflammatory, accumulate in lesions preferentially, and differentiate. Here we hypothesized that the bone marrow outsources the production of Ly-6C<sup>high</sup> monocytes during atherosclerosis.

**Methods and Results** - Using murine models of atherosclerosis and fate-mapping approaches, we show that hematopoietic stem and progenitor cells (HSPC) progressively relocate from the bone marrow to the splenic red pulp where they encounter GM-CSF and IL-3, clonally expand, and differentiate to Ly-6C<sup>high</sup> monocytes. Monocytes born in such extramedullary niches intravasate, circulate, and accumulate abundantly in atheromata. Upon lesional infiltration, Ly-6C<sup>high</sup> monocytes secrete inflammatory cytokines, reactive oxygen species, and proteases. Eventually, they ingest lipids and become foam cells.

**Conclusions** - Our findings indicate that extramedullary sites supplement the bone marrow’s hematopoietic function by producing circulating inflammatory cells that infiltrate atherosclerotic lesions.

**Key words:** Atherosclerosis, Imaging, Immune System, Immunology, Macrophage
Monocytes are myeloid leukocytes that circulate in the blood and patrol the vascular endothelium. During inflammatory diseases, monocytes accumulate in target sites and mature to macrophages or dendritic cells. Although monocytes are thought to arise exclusively in the bone marrow, hematopoietic stem and progenitor cells (HSPC), which are developmentally upstream, readily mobilize from their bone marrow niches, accumulate in the periphery, and differentiate. While this phenomenon of extramedullary hematopoiesis is known to give rise to erythrocytes, platelets, granulocytes, and dendritic cells, it remains unknown whether HSPC can yield monocytes outside of the bone marrow. Likewise, the mechanisms that govern HSPC proliferation and differentiation, as well as the eventual fate of the various progeny, remain elusive.

Atherosclerosis is a chronic disease characterized by the accumulation of lipids and leukocytes in the arterial vessel wall. Among leukocytes, monocytes are essential to the disease’s development and exacerbation. Upon lesional accumulation, and as a consequence of ingesting lipids abundantly, monocyte-derived macrophages become foam cells, the key culprits of atherosclerotic complications. Of the two recognized murine monocyte subsets, Ly-6C high monocytes have been shown to accumulate preferentially in growing lesions and give rise to macrophages in atheromata. Ly-6C high monocytes are also believed to convert to Ly-6C low monocytes, but this conversion is compromised during atherosclerosis. It remains unknown whether extramedullary hematopoiesis in general, and the extramedullary production of Ly-6C high monocytes specifically, contributes to the development of atherosclerosis.

In this study, we focused on experimental atherosclerosis to determine whether lesion-accumulating monocytes can have extramedullary origins. Our data show that the spleen, which
contains a reservoir of undifferentiated monocytes in the steady state\textsuperscript{16-18}, becomes monocytopoietic during atherosclerosis.

\textbf{Methods}

\textbf{Animals}

C57BL/6J (wt), B6.SJL-PtprcaPep3\textsuperscript{b}/BoyJ (CD45.1\textsuperscript{+}), C57BL/6-Tg(UBC-GFP)30Scha/J (GFP\textsuperscript{+}), B6.Cg-Tg(ACTB-mRFP1)1F1Had\textsuperscript{+}/J (RFP\textsuperscript{+}), ApoE\textsuperscript{−/−} mice (B6.129P2-Apo\textsuperscript{em1Unc}), LDLR\textsuperscript{−/−}ApoB48\textsuperscript{−/−} (B6;129S-Apo\textsuperscript{b}Ldl\textsuperscript{mm1Her}) male and female mice were purchased from Jackson Laboratories. All protocols were approved by the Animal Review Committee at Massachusetts General Hospital. More details are described in the online-only Data Supplement.

\textbf{Animal models and in vivo interventions}

Splenectomy, spleen transplantation, parabiosis, and adoptive transfer of cells are described in detail in Supplemental Experimental Procedures. Mice were i.v. injected with blocking antibodies, with clodronate liposomes or with oxLDL, as described in online-only Data Supplement.

\textbf{Flow Cytometry}

Antibodies used in the study are listed in the online-only Data Supplement. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v8.8.6 (Tree Star, Inc.). Cells were sorted on a BD FACS\textsuperscript{Aria II} (BD Biosciences).

\textbf{Histology}

Aortae and spleens were excised, embedded in O.C.T. compound (Sakura Finetek), and flash-frozen in isopentane and dry ice. 5 μm-thick frozen sections were used in all staining.
protocols. Immunofluorescence staining was carried out using protocols described in the online-only Data Supplement.

**Intravital Microscopy**

Intravital microscopy was performed on exteriorized spleens of live animals. Time-lapse images were captured to visualize the behavior of cells. Details of the procedure are described in the online-only Data Supplement.

**Statistics**

Results were expressed as mean ± SEM. Statistical tests included unpaired, 2-tailed Student's t test using Welch's correction for unequal variances and 1-way ANOVA followed by Bonferroni Comparison Test. P values of 0.05 or less were considered to denote significance.

**Results**

**The spleen contributes Ly-6C<sup>high</sup> monocytes to the growing atheroma**

We have previously shown that the spleen contains a monocyte reservoir that mobilizes in response to acute injury<sup>17</sup>, but the role of this reservoir in chronic inflammatory diseases such as atherosclerosis is unknown. We chose to investigate a possible link between the splenic reservoir and atherosclerosis in ApoE<sup>−/−</sup> mice. Immunofluorescent staining, which provides information on spatial distribution, showed expansion of CD11b<sup>+</sup> cells throughout the red pulp of ApoE<sup>−/−</sup> mice (Figure 1A), indicating enlargement of this reservoir. Enumeration of splenic leukocytes in wild type C57BL/6 mice and ApoE<sup>−/−</sup> mice consuming a diet high in fat and cholesterol (HCD) revealed dramatic differences between the groups: ApoE<sup>−/−</sup> mice had a marked increase in myeloid but not lymphoid cell number (online-only Data Supplement Figure Ia, b), a finding that complements previous work<sup>12</sup>. Within the myeloid compartment of the
spleen, both monocyte and neutrophil numbers increased (online-only Data Supplement Figure Ic). We also detected an expansion of monocytes and neutrophils in the spleens of LDLR<sup>−/−</sup> ApoB<sub>48</sub><sup>−/−</sup> mice consuming Paigen’s diet, indicating that the phenomenon is ApoE independent (online-only Data Supplement Figure Id).

To address whether the spleen mobilizes monocytes during atherogenesis, we transplanted spleens from CD45.1 ApoE<sup>−/−</sup> mice to recently-splenectomized 30 week old CD45.2 ApoE<sup>−/−</sup> mice that had consumed a HCD for 20 weeks. The transplantation procedure anastomoses splenic and recipient vessels, preserves blood flow and organ integrity, and does not alter the relative proportion of endogenous leukocytes in the spleen, bone marrow and blood (online-only Data Supplement Figure Ie). The transplanted spleens resembled endogenous spleens in size and cellularity, had the characteristic purplish red color and were well perfused (online-only Data Supplement Figure If). Spleens of CD45.1 ApoE<sup>−/−</sup> mice consuming HCD for 20 weeks were large and enriched with myeloid cells (Figure 1B). As a control, we also transplanted spleens from CD45.1 ApoE<sup>−/−</sup> mice consuming a chow diet (chow) because they contained small spleens and relatively small myeloid reservoirs (Figure 1B), and spleens from CD45.1 ApoE<sup>−/−</sup> mice consuming HCD that had their reservoirs depleted with clodronate liposomes (Figure 1B).

Transplantation of CD45.1 ApoE<sup>−/−</sup> mice consuming HCD for 20 weeks led to a large accumulation of spleen-derived monocytes, but not macrophages, in the recipient blood (4.7 × 10<sup>5</sup> ± 2 × 10<sup>5</sup> monocytes in blood) (Figure 1C). After 24 hours, 45 ± 15% of the monocytes found in the blood were splenic-derived. Although we focused our analysis on monocytes and progeny (CD11b<sup>+</sup>Lin<sup>−</sup>, Figure S1A), other cells such as neutrophils also accumulated (data not shown). In accordance with the blood data, we observed a substantial population of CD45.1<sup>+</sup>
cells in the recipient aorta consisting of F4/80<sup>low</sup> monocytes and F4/80<sup>high</sup> macrophages (Figure 1D), thus indicating that spleen-experienced F4/80<sup>low</sup> monocytes accumulated in lesions and matured to F4/80<sup>high</sup> macrophages locally. The observed contribution of 19% in 24 hours is an underestimation given that an endogenous pool of macrophages already resides in the aorta prior to transplantation. By focusing on monocytes in the aorta that had recently proliferated we estimated that the spleen contributed ~30% of monocytes in 1 day. Remarkably, 100% of monocytes arriving from the spleen (i.e., CD45.1) were Ly-6C<sup>high</sup> whereas monocytes arriving from all other sources (i.e., CD45.2) contained a mixture of Ly-6C<sup>high</sup> and Ly-6C<sup>low</sup> monocytes. Transplantation of spleens from either of the two controls led to a very low accumulation of monocytes and progeny in the aorta (Figure 1E). Importantly, the total number of aortic monocytes was similar in ApoE<sup>−/−</sup> HCD spleen recipients compared to age and diet matched controls that did not receive a spleen by transplantation (online-only Data Supplement Figure Ig), indicating that the transplantation procedure did not interfere with cell accumulation in the aorta. The finding is expected because the transplantation experiments consisted of removing the endogenous spleen, and thus represented splenic exchange rather than supplementation. The intima of aortic root lesions contained spleen-derived myeloid cells of mixed morphology readily identified by CD45.1 immunofluorescence (Figure 1F). The spleen therefore contributes inflammatory Ly-6C<sup>high</sup> monocytes to the growing atheromata.

**Spleen-experienced monocytes express pro-IL-1β, have proteolytic capacity, contribute reactive oxygen species, and give rise to lipid-laden macrophages in atheromata**

Monocyte-derived macrophages in atheromata secrete inflammatory cytokines, express proteolytic enzymes, contribute reactive oxygen species, and ingest lipids. Each of these functions profoundly influences lesion evolution: inflammatory cytokines and reactive oxygen
species propagate inflammation, proteolysis remolds the extracellular matrix, and lipid uptake yields foam cells. We therefore sought to determine whether spleen-experienced monocytes and macrophages exhibit these properties. We compared spleen-experienced monocytes and their descendent macrophages to monocytes and macrophages that arrived from all sources by transplanting CD45.1+ apoE−/− spleens to CD45.2+ apoE−/− animals for 2 days, as shown in Figure 1. For simplicity, we will call these spleen-experienced monocytes and their descendent macrophages “splenic” while those arriving from all sources “medullary”. It is important to note, however, that at least some of the “medullary” cells might have experienced the spleen before accumulating in lesions.

Evaluating inflammatory cytokine expression focused on IL-1β, a monocyte product implicated in many aspects of atherogenesis. Aortic cells in ApoE−/− HCD mice expressed a higher proportion of pro-IL-1β+ compared to wt controls (online-only Data Supplement Figure IIa, b), reflecting these animals’ higher inflammatory burden. Spleen transplantation revealed similar pro-IL-1β expression among splenic (CD45.1+) and medullary (CD45.2+) monocytes, suggesting that splenic monocytes’ inflammatory capacity is comparable to their counterparts’ (Figure 2A). Splenic (CD45.1+) aortic macrophages remained pro-IL-1β+ at similar proportions as monocytes, but at higher proportions than CD45.2+ macrophages. This indicates that aortic monocytes and macrophages of splenic origin are at least as, if not more, inflammatory as their CD45.2+ counterparts. To investigate proteolysis, we injected spleen recipients with a protease-activatable fluorescent sensor that reports on cysteiny1 cathepsin activity in vivo. At the cellular level, most lesional monocytes and macrophages of splenic origin (CD45.1+) exhibited proteolytic activity, and comparison to lesional monocytes and macrophages accumulating from the bone marrow (CD45.2+) revealed similar proportion (83% vs 78%; 98% vs 85% were
positive for Prosense-680) of signal (Figure 2B). Monocytes had less proteolytic activity when residing in the bone marrow and spleen, indicating that proteolysis coincides with activation in destination sites (online-only Data Supplement Figure IIc). To measure cellular redox states we utilized an intracellular probe that measures oxidative stress.\textsuperscript{22} Regardless of origin, monocytes and macrophages expressed reactive oxygen species, although macrophages derived from spleen-experienced monocytes expressed them at somewhat lower levels (Figure 2C). Finally, we asked whether spleen-experienced monocytes can give rise to lesional foam cells. Circulating monocytes of either origin accumulated DiI-oxLDL to the same extent (online-only Data Supplement Figure IId) and aortic atheromata contained large myeloid cells of splenic origin that took up Oil-Red-O (Figure 2D, E and online-only Data Supplement Figure IIe), indicating that the spleen contributes foam cell precursors.

The spleen is a secondary lymphoid organ that supports multiple functions: It contains T and B cells that participate in adaptive immunity, and macrophage and dendritic cell subsets that scavenge erythrocytes and screen for blood borne infections.\textsuperscript{23} In animal models, splenectomized mice\textsuperscript{24,25} as well as hamsters depleted of monocytes with clodronate liposomes, develop larger lesions.\textsuperscript{26} We therefore wondered whether and how splenectomy alters the lesional monocyte/macrophage/foam cell content. As expected, lesions in female mice splenectomized for 12 weeks were larger (Figure 2F) as assessed by H&E staining (online-only Data Supplement Figure IIf). However, the lesions appeared to be less cellular, prompting us to evaluate their content with macrophage markers. Staining for Mac3, CD11b, and F4/80 revealed that aortic root sections in splenectomized mice contained fewer monocyte/macrophages (Figure 2G, H and online-only Data Supplement Figure IIg) but had larger accellular areas (Figure 2I). Accordingly, cellular ORO areas were smaller in splenectomized mice (Figure 2H, J). We
detected no differences in collagen content, as measured by Masson Trichrome (online-only Data Supplement Figure IIh) and no differences in smooth muscle cell content (online-only Data Supplement Figure IIIi). Flow cytometry of digested aortas confirmed that lesions of splenectomized mice contained fewer monocytes/macrophages (Figure 2K, L). Our studies are in accordance with the observation that, when monocyte supply is reduced, lesions become larger and less cellular\textsuperscript{26}. Thus, the spleen provides a surplus of cells that serve to shape the evolving lesion.

**The spleen contains proliferating myeloid cell progenitors that give rise to their progeny in vivo**

The cell tracking experiments described thus far may simply reflect production of monocytes in the bone marrow, their circulation through the spleen, and eventual accumulation in the lesions. We therefore sought to determine whether the spleen can produce monocytes from progenitors. In vitro, splenocytes of ApoE\textsuperscript{−/−} HCD mice formed many more granulocyte/macrophage colonies than wt controls (Figure 3A), indicating clonal myeloid-cell proliferation. Compared to wt controls, ApoE\textsuperscript{−/−} HCD mice contained numerous HSPC, including LSK (Lin\textsubscript{2}–Sca-1\textsuperscript{+} c-kit\textsuperscript{+}) cells and common myeloid progenitors (CMP), as well as granulocyte and macrophage progenitors (GMP), which are the most committed progenitors known to give rise to monocytes and neutrophils\textsuperscript{4} (Figure 3B and online-only Data Supplement Figure IIIa). Common lymphoid progenitors (CLP) did not increase in ApoE\textsuperscript{−/−} HCD mice, indicating a preference toward the myeloid lineage in the spleen (online-only Data Supplement Figure IIIa). GMP enumeration over 30 weeks of diet showed continued growth in the spleen. The bone marrow’s GMP population also grew, but less markedly and transiently (Figure 3C). Indeed, in many older animals the spleen’s GMP population exceeded the bone marrow’s.
Notably, while the spleen also contained macrophage and dendritic cell progenitors (MDP)\textsuperscript{27}, the aorta and para-aortic lymph nodes were virtually devoid of HSPC (data not shown). In keeping with the expanded reservoir, the spleens of LDLR\textsuperscript{−/−}ApoB48\textsuperscript{−/−} mice also contained higher numbers of GMPs (online-only Data Supplement Figure IIIb). These findings demonstrate that the spleens of atherosclerotic mice contain the requisite myelopoietic cells.

To test for monocytopoiesis in vivo, we injected $10^5$ highly purified green fluorescence protein (GFP)-expressing GMP (online-only Data Supplement Figure IIIc) into either ApoE\textsuperscript{−/−} HCD or wt mice and enumerated GFP\textsuperscript{+} cells 5 days later. Considerably more GFP\textsuperscript{+} cells accumulated in the spleens of ApoE\textsuperscript{−/−} HCD mice than the controls (Figure 3D, E). Among these cells we detected progenitors and differentiated CD11b\textsuperscript{−}Gr1\textsuperscript{+} monocytes and neutrophils (online-only Data Supplement Figure IIIId), which indicates GMP expansion. The number of GFP\textsuperscript{+} cells accumulating in the bone marrow, however, was similar in wt and ApoE\textsuperscript{−/−} HCD mice (Figure 3E). This similarity likely reflects a preference for mobilized progenitors to seed the spleen in atherosclerosis (progenitors were injected i.v.).

To demonstrate that the spleen contributes rather than simply collects monocytes we performed spleen transplantation experiments for longer durations. The monocyte half-life is estimated at a few hours to 1-2 days\textsuperscript{11,28}, and adoptive transfer of $5 \times 10^6$ sorted monocytes by a procedure that allows their retrieval 1 day later\textsuperscript{12} failed to yield any cells in lesions, blood or spleen 5 days later. We therefore reasoned that if monocytes and their progenitors reside in the spleen briefly or simply circulate through its fenestrated parenchyma, spleen transplantation would result in a rapid and complete cell turnover. If, however, the spleen produces monocytes, it should maintain progenitors and monocytes from the original animal for a period exceeding 2 days. In accordance with the second possibility, CD45.2\textsuperscript{+} ApoE\textsuperscript{−/−} HCD mice that received
CD45.1+ ApoE–/– spleens 10 days earlier contained 3.2 × 10^6 GMPs, of which 25% were still CD45.1+ (Figure 3F), and many were proliferating (Figure 3G). The aortas of these mice also contained numerous CD45.1+ monocytes and macrophages (Figure 3H). Importantly, the 75% of GMPs that were CD45.2+ in these animals indicates that the bone marrow continuously supplies and replenishes the splenic progenitor pool. In contrast, transplantation of naive spleens, which do not contain progenitors, resulted in complete turnover of the monocyte pool in 2 days. Thus, if the bone marrow is the upstream source of hematopoietic cells, the spleen is a seeding ground for an amplification cascade during inflammation. Together, our findings indicate that the spleen contains proliferating myeloid cell progenitors that give rise to their progeny in vivo.

**Splenic monocytopoiesis gives rise to lesion-infiltrating monocytes**

Having established that the spleen contains proliferating hematopoietic progenitors, we next sought to determine more directly whether the spleen supports monocytopoiesis. We injected GFP+ GMPs into age and diet-matched spleen-containing and asplenic ApoE–/– HCD mice. Compared to controls, asplenic recipients accumulated fewer GFP+ CD11b+ cells in the blood and aorta (Figure 4A, B). In the aorta, among the GFP+ cells, we found differentiated monocytes, mature macrophages, but not progenitors, which would appear in the lower-left quadrant (CD11b– MHCII/CD11c/F4/80–) (Figure 4C), indicating a direct and spleen-dependent link from hematopoietic progenitors to tissue descendants. We confirmed these flow cytometry tracking experiments by detecting adoptively transferred CD45.1+ F4/80+ cells microscopically on tissue sections (Figure 4D).

To confirm the role of the spleen in maintaining the aortic pool of monocytes, we joined CD45.1 to CD45.2 mice by parabiosis (Figure 4E), a technique that establishes a shared circulation by which bone marrow HSPC can seed partner tissues.5, 6, 17, 28 Two weeks after
parabiont separation, the percent of myeloid cell chimerism in wt aortas was low (~5%) and spleen-independent (Figure 4E). This finding supports the observation that under normocholesterolemic conditions the spleen does not produce monocytes. In contrast to wt mice, the aortas of spleen-containing ApoE−/− HCD animals showed a 3-fold increase in chimerism. Strikingly, splenectomy decreased this chimerism by nearly 50%. These data demonstrate, by an independent approach, that the spleen contributes myeloid cells to the atherosclerotic aorta.

**GM-CSF and IL-3 promote survival and proliferation of progenitor cells and their progeny**

The growth factors GM-CSF and IL-3 can stimulate hematopoiesis, particularly emergency hematopoiesis, but their role in atherosclerosis is poorly understood. Recent observations in experimental atherosclerosis that HSPCs increase the common receptor subunit for GM-CSF and IL-3, prompted us to investigate the influence of these cytokines on myelopoiesis. GM-CSF and IL-3-producing cells increased preferentially in the spleen during atherosclerosis (Figure 5A). In the spleen, the red pulp of ApoE−/− HCD mice contained numerous GM-CSF and IL-3-producing cells that stained specifically for the growth factors on tissue sections (Figure 5B and online-only Data Supplement Figure IVa) and co-stained for the leukocyte marker CD45 by flow cytometry (online-only Data Supplement Figure IVb).

Negligible levels of GM-CSF and IL-3 cells were detected in the steady-state spleens. Colony forming assays showed that GM-CSF and IL-3 were sufficient to drive myelopoiesis of splenic progenitors (online-only Data Supplement Figure IVc). We therefore injected antibodies against GM-CSF and IL-3 on 5 consecutive days and evaluated the myeloid lineage in the spleen, bone marrow and blood in vivo using four approaches. First, we analyzed splenic GMPs. Neutralization of IL-3, GM-CSF or both increased the proportion of GMPs undergoing apoptosis.
in situ (as measured by DNA content) and correspondingly decreased their total number in the spleen (Figure 5C). Second, we performed pulse-chase experiments which, as in those depicted in Figures 3 and 4, involved the transfer and retrieval of GFP+ cells. Neutralization of IL-3, GM-CSF or both prevented the appearance of numerous myeloid cells in the spleen and blood (Figure 5D). Third, we evaluated the survival and proliferation of mature myeloid cells in the spleen. Again, neutralization increased the proportion of in situ dying monocytes and neutrophils and decreased their local proliferation (Figure 5E). Finally, we evaluated the endogenous number of myeloid cells in spleen and blood and found that neutralization attenuated splenic and blood monocytosis and neutrophilia by up to 80% (Figure 5F). In vitro confirmatory experiments showed that myelopoiesis was attenuated with only a single dose of GM-CSF and IL-3 neutralizing antibodies (Figure 5G). Expectedly, the dramatic decrease in vivo resulted not only from local effects on the spleen, but also from effects on the bone marrow (online-only Data Supplement Figure IVc). These data demonstrate that GM-CSF and IL-3 orchestrate the survival and proliferation of myeloid progenitors and their descendants in the bone marrow and spleen in atherosclerosis.

**Extramedullary hematopoiesis occurs in response to peritoneal endotoxin challenge and can be visualized in vivo**

Experiments thus far have shown that in experimental atherosclerosis: (i) HSPC accumulate in the spleen and proliferate in response to GM-CSF and IL-3, and (ii) the spleen contributes monocytes to the growing atheromata. We next sought to determine whether the process occurs in a different model and whether it can be visualized in vivo. Mice that received LPS daily for 4 days developed visibly larger spleens (online-only Data Supplement Figure Va) containing plenty of monocytes (11-fold expansion over steady state) and neutrophils (23-fold
expansion; online-only Data Supplement Figure Vb), that resided in the red pulp (online-only Data Supplement Figure Vc). To evaluate whether the spleen contributes newly-made neutrophils and monocytes in this model, we first enumerated hematopoietic progenitor cells. In response to LPS, GMPs expanded in the spleen (~62-fold) (online-only Data Supplement Figure Vd). The progenitors proliferated vigorously (47% were in S or G₂ phase of the cell cycle at a given time) (online-only Data Supplement Figure Ve) and gave rise to granulocyte-macrophage colony forming units (online-only Data Supplement Figure Vf).

We therefore tested whether splenic GMPs give rise to their progeny. Pulse-chase experiments involving i.v. transfer of GFP GMPs or red fluorescent protein (RFP)-expressing GMPs to non-irradiated (wt) C57BL/6 mice that were naive or that received LPS permitted us to track cell fate by flow cytometry and intravital microscopy. GMP injected into naive mice gave rise to only a few progeny (Figure 6A). Injection of GMP to mice that received LPS, in contrast, led to marked cellular expansion. Three days after transfer, the fluorescent (in this case, GFP⁺) cells in the spleen were still mostly CD11b⁻ Gr1⁻ progenitors, although a few fluorescent neutrophils and a few Ly-6C<sup>hi</sup> monocytes (CD11b⁺ Gr1<sup>+</sup> cells) emerged in the circulation (Figure 6A). Three to five days later (day 6-8), the fluorescent cells in the spleen and blood were almost exclusively CD11b⁺ Gr1<sup>+</sup> neutrophils with a few monocytes, but not progenitors, mature macrophages or dendritic cells (Figure 6A, B). Enumeration revealed significant expansion of neutrophils and monocytes in response to LPS in the spleen and blood (Figure 6C). The bone marrow contained numerous GFP progeny, but, as reported in the atherosclerosis model, there was no increase in their number in response to LPS. These data show that escalation of the neutrophil and monocyte response in response to endotoxin associates with an increase of biologically active progenitors in the spleen.
Intravital microscopy showed that GMP gave rise to clusters of cells that resided outside of blood vessels in the red pulp (Figure 6D). In this pulse-chase experiment, clusters appeared 3 days after adoptive transfer, increased in size by 8 days, and disappeared by 12 days (online-only Data Supplement Figure Vg, h), which is in keeping with the expected life-span of GMP. GFP GMPs and RFP GMPs co-injected in equal numbers (Figure 6E) gave rise to clusters that were exclusively red or exclusively green, indicating that GMP seeded the spleen, proliferated clonally, and differentiated locally (Figures 6F and online-only Data Supplement Vi-k). Time-lapse imaging of representative clusters showed that locally produced cells can intravasate and thus contribute to the systemic repertoire (Figure 6G). To enumerate this contribution, mice were subjected either to splenectomy (Figure 6H) or to spleen transplantation (Figure 6I). Both approaches revealed that a substantial number of myeloid cells accumulating in the peritoneum were of splenic origin. The cells were inflammatory as evidenced by the expression of TNFα (Figure 6J). Altogether, these data reveal that extramedullary sites such as the spleen can produce circulating monocytes and neutrophils that accumulate in their respective inflammatory destinations. The model which describes the study’s findings is shown in Figure 7.

Discussion

The motivation for the current study rested on two landmark papers published in the 1960s. The first laid the foundation that HSPC circulate in the blood rather than simply reside in the bone marrow, whereas the second argued that, in the steady state, the bone marrow is the exclusive monocyte production site. Multiple studies since have enriched our understanding of these processes. Extramedullary hematopoiesis occurs in development and in a number of genetic and myeloproliferative conditions. It involves several discrete steps: first, bone
marrow HSPC mobilize; second, mobilized HSPC seed extramedullary sites; third, seeded HSPC proliferate and mature. The cell types that have been described to arise through extramedullary hematopoiesis are terminally-differentiated, and many are tissue-resident. Monocytes, on the other hand, are intermediate, circulating cells, developmentally downstream of HSPC but upstream of dendritic cells and macrophages. Our study places extramedullary hematopoiesis in a larger context not only because it shows that a GM-CSF and IL-3-rich splenic environment can produce monocytes and neutrophils that then circulate, but also because it illustrates an extramedullary cascade in which HSPC proliferation, differentiation and terminal maturation are compartmentalized in different organs. In atherosclerosis, our data imply, the bone marrow outsources the production of circulating leukocytes.

Of all the organs, the spleen may be an ideal outsource destination. The organ has an open circulation, allowing for fast exchange with the blood, yet it is capable of cell retention through a myriad of adhesive ligands. The organ can accommodate vast quantities and fluctuations of cells, especially in the red pulp. It also allows for rapid exit of undifferentiated monocytes, indicating that entry into its parenchyma neither forces differentiation nor precludes recirculation. Thus, location, elasticity and architecture render the spleen a perfect seeding ground for the emergency production of inflammatory cells.

It is unknown whether extramedullary hematopoiesis is important in the development of human atherosclerosis. The extent to which the spleen contributes to disease, however, has received some attention. The organ may be dispensable, but it is not unimportant. In humans, splenectomy heightens the risk of infection and ischemic heart disease, probably due to multiple mechanisms involving platelets, B cells, T cells and many of the other components that constitute the organ. The observations made here in response to hypercholesterolemia or endotoxin
indicate a role for the spleen in supplying Ly-6C\textsuperscript{high} monocytes, which are known to be inflammatory and proteolytic. We show that, by eliminating this splenic source, lesions become less cellular although how this particular profile translates to lesion stability requires further study. Future studies will also need to elucidate how specific splenic functions can be targeted therapeutically and how other functions, such as the protective function of B1 B cells\textsuperscript{24} can be spared.

That extramedullary generation of Ly-6C\textsuperscript{high} monocytes occurs during atherosclerosis, when hypercholesterolemia forces a continuous leukocyte supply, is worth further discussion. The steady-state spleen houses a reservoir of undifferentiated monocytes which are, in the absence of neighboring splenic HSPC, bone marrow-derived. Extramedullary monocytopoiesis’ contribution during inflammation may be exclusively numerical: with repetitive demand the organism outsources the production of otherwise identical cells. Alternatively, splenic monocytes, produced in an inflammatory GM-CSF and IL-3-rich environment, may differ qualitatively from their medullary counterparts. In the present study we show that monocytes born in extramedullary sites belong to the inflammatory subset, express cytokines, reactive oxygen species, and proteases. They are thus functional and are known to promote the generation of lesions. Future studies will need to determine whether splenic birth or experience influences these cells in additional ways.

HSPC mobilization is important to immunosurveillance and likely plays a role in bone-marrow niche reconstruction\textsuperscript{5,45} HSPC differentiation in extramedullary sites may be essential to the replenishment of dendritic cells and macrophages\textsuperscript{46} The extramedullary HSPC production of circulating cells, we now show, contributes importantly to inflammatory diseases. This shift in
the hematopoietic topographical hierarchy during inflammation is likely to have significant biological, diagnostic, and therapeutic implications.

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References:


Figure Legends:

**Figure 1.** Splenic myeloid cells infiltrate atherosclerotic lesions. **A.** Immunofluorescence (IF) of CD11b cells (red) in spleens of C57BL/6 (wt) and apoE<sup>−/−</sup> mice consuming a high cholesterol diet (HCD) for 20 weeks. Data show that the red pulp myeloid component enlarges during atherosclerosis and pushes the white pulp clusters away from each other. **B.** Size of the monocyte reservoir in apoE<sup>−/−</sup> mice consuming a chow diet for 20 weeks, apoE<sup>−/−</sup> mice consuming HCD 20 weeks, and apoE<sup>−/−</sup> mice consuming HCD for 20 weeks and then injected with clodronate liposomes 1 day prior (n = 2-10). **C.** Presence of CD45.1<sup>+</sup> cells in the blood of CD45.2<sup>+</sup> mice that received CD45.1<sup>+</sup> spleens by transplantation. **D.** Spleen transplantation from CD45.1 apoE<sup>−/−</sup> donors consuming HCD to CD45.2 apoE<sup>−/−</sup> recipients. Data show direct accumulation and differentiation of splenic Ly-6C<sup>high</sup> monocytes in aortic lesions in 1 day. One of 11 representative experiments is shown. **E.** Spleen transplantation from CD45.1 apoE<sup>−/−</sup> donors consuming chow and from CD45.1 apoE<sup>−/−</sup> donors consuming HCD and then injected with clodronate liposomes. Data show negligible accumulation of splenic monocytes in aortic lesions in these controls. **F.** IF on the aortic root with antibodies against CD45.1 (green), CD11b (red) and the merge of the two (yellow). DAPI depicts nuclei (blue). Arrows point to CD11b<sup>+</sup> cells of splenic origin. For all flow cytometric plots the ticks represent 0, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup> mean fluorescence units (MFI), except for axes labeled “cell no.” or “SSC” where the ticks represent 0, 50K, 100K, 150K, 200K and 250K MFI.

**Figure 2.** Splenic cells are inflammatory and shape lesional evolution. **A.** Spleen transplantation from CD45.1<sup>+</sup> apoE<sup>−/−</sup> to CD45.2<sup>+</sup> apoE<sup>−/−</sup> mice. Expression of pro-IL-1β on monocytes and
macrophages directly ex vivo (i.e., unstimulated). Contour plots show pro-IL-1β expression gated on lesional monocytes and macrophages of splenic (CD45.1+, red) or other source (i.e., bone marrow) (CD45.2+, blue) origin. The control density plots (black) represent isotype controls. **B.** Contour plots show protease activity gated on lesional monocytes and macrophages of splenic (CD45.1, red) or other source (i.e., bone marrow) (CD45.2+, blue) origin. The control density plots (black) are gated on all leukocytes. **C.** Contour plots show presence of reactive oxygen species on lesional monocytes and macrophages of splenic (CD45.1+, red) or other source (i.e., bone marrow) (CD45.2+, blue) origin. The control density plots (black) are gated on cells that did not receive the probe. Representative plots in A-C of at least 2 independent experiments are shown. **D.** Spleen transplantation for 10 days. Data show IF on the aortic root with antibodies against CD45.1 (splenic cells, green), F4/80 (macrophages, red), and the merge (yellow). DAPI depicts nuclei (blue). Arrows point to F4/80+ cells of splenic origin. **E.** Oil red O (ORO) staining on the same section as in D shows colocalization of ORO with spleen-derived macrophages. **F.** Splenectomy of apoE−/− HCD mice for 12 weeks. Data show enumeration of total lesion size using H&E. **G.** Representative Mac3 expression on aortic root sections in control and splenectomized apoE−/− HCD mice. **H.** Enumeration of CD11b, ORO, F4/80, and Mac3 areas on aortic root sections in control and splenectomized apoE−/− HCD mice. **I.** Enumeration of the necrotic core size in the same groups as above. **J.** Representative ORO staining in the same groups as above. **K.** Flow cytometry of digested aortas. Dot plots show cellular distribution from control and splenectomized apoE−/− HCD mice. The monocyte/macrophage gate is shown. **L.** Total number of monocytes and macrophages/DC enumerated by flow cytometry (means ± SEM, n = 5-11) *P< 0.05.
**Figure 3.** The spleen contains proliferating myeloid cell progenitors that give rise to their progeny in vivo. **A.** CFU-GM shows colony formation in spleens of wt and apoE<sup>−/−</sup> HCD mice (means ± SEM, n = 4). *P < 0.05. **B.** Phenotypic analysis of granulocyte and macrophage progenitors (GMP) in spleens of wt and apoE<sup>−/−</sup> HCD mice. **C.** Enumeration of GMP in spleens and bone marrow of apoE<sup>−/−</sup> mice fed HCD for up to 30 weeks. Linear regression was performed. **D.** Adoptive transfer of GFP<sup>+</sup> GMP to wt and apoE<sup>−/−</sup> HCD mice. Data show GFP cells in spleens 5 days after transfer. **E.** Enumeration of data above. Data show the fold increase from wt to apoE<sup>−/−</sup> HCD mice of adoptively transferred GFP<sup>+</sup> cells in the bone marrow (BM) and spleen (data shown are pooled from two independent experiments). **F.** CD45.1<sup>+</sup> spleens from apoE<sup>−/−</sup> HCD mice were transplanted to asplenic CD45.2<sup>+</sup> apoE<sup>−/−</sup> HCD mice for 10 days. Data show chimerism of GMP in spleens 10 days after transplantation. **G.** Cell cycle analysis of CD45.1<sup>+</sup> and CD45.2<sup>+</sup> GMP in transplanted spleens shown in G. Numbers indicate percentage of cells in S/G2/M phase (means ± SEM, n = 4). **H.** Monocyte accumulation in aortic tissue of the mice described in F and G. Representative of 2 independent experiments are shown.

**Figure 4.** Splenic monocytopoiesis gives rise to lesion-infiltrating monocytes. **A.** Adoptive transfer of GFP<sup>+</sup> GMP to apoE<sup>−/−</sup> HCD mice with or without their spleen. Data show GFP<sup>+</sup> CD11b cells retrieved from blood and aorta 5 days after transfer. **B.** Enumeration of data above (data shown are pooled from two independent experiments). *P < 0.05. **C.** Differentiation of GFP<sup>+</sup>GMPs into lesional macrophages. A representative contour plot shows that GFP<sup>+</sup> cells that were injected as GMP have accumulated in lesions as monocytes and matured to macrophages. **D.** CD45.1 GMP were adoptively transferred to apoE<sup>−/−</sup> HCD mice and the aorta was harvested 5 days after transfer. A representative pictograph shows CD45.1 (GMP-derived, green), F4/80
(macrophages, red) and the merge (yellow) in the intima. E. CD45.1 and CD45.2 wt or apoE<sup>+/−</sup> HCD mice were joined in parabiosis for 3 weeks, separated, splenectomized (or not) and assessed for chimerism 2 weeks later. Data show chimerism for CD11b<sup>+</sup> cells in the aorta (means ± SEM, n = 4).

**Figure 5.** GM-CSF and IL-3 control survival and proliferation of myeloid progenitors and progeny in atherosclerosis. A. Enumeration of GM-CSF and IL-3-producing cells by flow cytometry in the spleen and bone marrow in wt and apoE<sup>+/−</sup> HCD mice. Data show preferential increase of GM-CSF and IL-3 producing cells in the spleen (means ± SEM, n = 5). *P< 0.05. B. Presence of GM-CSF and IL-3-producing cells in the red pulp of wt and apoE<sup>+/−</sup> HCD mice. C. Effect of IL-3 and GM-CSF neutralization on endogenous splenic GMP. Data show percent of cells in subG<sub>1</sub> and total number of GMP in spleen (means ± SEM, n = 4-5). D. Effect of IL-3 and GM-CSF neutralization on development of myeloid cells after GFP<sup>+</sup> GMP pulse chase. Data show GFP<sup>+</sup> CD11b<sup>+</sup>Gr1<sup>+</sup> cells in the spleen and blood (means ± SEM, n = 4-5). E. Effect of IL-3 and GM-CSF neutralization on apoptosis and proliferation of endogenous monocytes and neutrophils in the spleen (means ± SEM, n = 4-5). F. Effect of IL-3 and GM-CSF neutralization on the endogenous CD11b<sup>+</sup>Gr1<sup>+</sup>repertoire in the spleen and blood (means ± SEM, n = 4-5). *P< 0.05 compared to wt (A, B) or HCD isotype (C, D, E, F). G. Granulocyte macrophage colony forming units in the spleen. Data show that, in the absence of cytokines, colonies do not form in the spleen, that GM-CSF and IL-3 are sufficient for colony formation, and that a single dose of anti-GM-CSF and anti-IL-3 attenuates colony formation in vitro (means ± SEM, n = 2).
Figure 6. Extramedullary hematopoiesis gives rise to monocytes in response to repeated peritoneal endotoxin challenge. A. Cartoon depicts a pulse-chase experiment in which GFP+ GMPs were adoptively transferred to wild type (wt) C57BL/6 mice that remained naive or were injected with LPS. A representative plot of at least three independent experiments is shown. B. GFP+ GMPs were adoptively transferred to wild type (wt) C57BL/6 mice injected with LPS. Data are representative of at least three independent experiments. C. Enumeration of GFP+ CD11b+Gr1+ cells adoptively transferred as GFP+ GMPs 8 days earlier and retrieved from host spleen and blood of naive or inflammatory mice (means ± SEM, n = 3 to 8). *P< 0.05. D. Intravital microscopy pictograms of the splenic red pulp depict clusters of GFP+ cells adoptively transferred i.v. 8 days earlier into inflammatory (LPS-injected) mice. Vasculature is shown in red and the scale is depicted with white bars. Data are representative of at least three independent experiments. E. Cartoon depicts experimental design for the co-injection of equal numbers of GFP+ GMPs and RFP+ GMPs into C57BL/6 mice injected with LPS. F. Green and red clusters in the subcapsular red pulp 5 days after injection of equal numbers of GFP+ GMPs and RFP+ GMPs. Vasculature is shown in blue and the scale is depicted by a white bar. Data are representative of at least three independent experiments. G. A prototypic departing cell is shown to intravasate and enter the circulation. H. Accumulation of myeloid cells in splenectomized animals. Mice received LPS and were either splenectomized or subjected to sham surgery. Four days later peritoneal CD11b+Gr1+ cells were enumerated. I. Spleen transplantation from CD45.1 donors to CD45.2 recipient mice. Donors were either naive or received LPS. The graph shows the total number of splenic CD11b+Gr1+ that had accumulated in the peritoneum. J. Expression of intracellular TNFα. Histograms show TNFα expression on stimulated cells gated on
CD11b^+Gr1^+ cells of splenic (CD45.1^+, red) or other source (i.e., bone marrow) (CD45.2^+, blue) origin. Data are representative of at least two independent experiments.

**Figure 7.** Model depicting the extramedullary generation of monocytes in inflammation. Model shows that mobilized hematopoietic stem and progenitor cells accumulate in the splenic red pulp and give rise to monocytes via IL-3 and GM-CSF. Spleen-derived monocytes infiltrate inflammatory sites and mature to macrophages. The splenic contribution increases as the reservoir enlarges. Monocytes that accumulate from bone marrow directly are omitted in this cartoon.
Extramedullary Hematopoiesis Generates Ly-6C<sup>high</sup> Monocytes that Infiltrate Atherosclerotic Lesions


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Supplemental Material

Expanded Methods and Results

Animals. The LDLR−/−ApoB48−/− mouse was selected because it is reported to more closely mimic human atherosclerosis than other models. ApoE−/− animals had been backcrossed to the C57BL/6 background for at least ten generations. ApoE−/− CD45.1+/+ were generated after backcrossing ApoE−/− mice to C57BL/6 CD45.1+/+. At 10 wk of age, ApoE−/− mice were placed on a Western diet (21.2% fat/weight; 0.2% cholesterol) (Harlan Teklad, Madison, WI) or remained on a chow diet for durations listed in the manuscript. The LDLR−/−ApoB48−/− mice were placed on Paigen’s high fat diet without cholate (Research Diets, New Brunswick, NJ) for 16 wk at 8 weeks of age. The remaining animals consumed a regular chow diet.

Animal models and in vivo interventions. Splenectomy: Under isofluorane anesthesia, the peritoneal cavity of mice was opened and the splenic vessels were ligated using a 6.0 silk suture. The spleen was then carefully removed. For control experiments, the peritoneum was opened, but the spleen was not excised. The procedure itself does not change the number of circulating monocytes and neutrophils in the steady state. Spleen transplantation: Spleen transplantation was conducted as previously described. Briefly, spleen donor mice were anesthetized with a subcutaneous injection of ketamine (90 mg/kg) and xylazine (10 mg/kg), followed by an intravenous injection of 200 units of heparin (American Pharmaceutical, Schaumburg, II). The complete inhibition of clotting ensures that no vascular or intrasplenic thrombosis occurs. In deep anesthesia, the thorax was then opened and the right atrium sectioned to allow blood to exit during perfusion. Over a period of 3 minutes, the entire mouse was then perfused with a total of 15 ml of normal saline through a 25G needle inserted into the apex of the left ventricle. At the end of this procedure, fluid exiting the right atrium was clear which indicates thorough removal of the donor blood. Under isofluorane (1-2%), supplemented with oxygen (1-2 L) anesthesia, the abdomen of the recipient mouse was then opened with a longitudinal incision. The pancreas, the spleen and the abdominal vasculature in the epigastric region were identified and ligated with 6.0 cotton (Ethicon). The celiac artery was then isolated, and the hepatic and gastric artery ligated with 10.0 suture (Ethicon). The abdominal aorta was ligated and cut just below the celiac artery with micro-dissection scissors (ROBOZ, Rockville, MD), and also dissected above the celiac artery. This approach resulted in an aortic cuff.
connected to the splenic artery, which allowed vascular anastomosis of the spleen to the recipient. Following ligation of the bile duct, the portal vein was isolated, and the superior and inferior mesenteric and gastric veins were ligated. The portal vein was intersected closely to the liver. The entire organ package containing the vascular connections, spleen and the pancreas was then removed and stored in ice-cold saline for 15 minutes while the recipient was prepared. The recipient (CD45.1) was anesthetized with isofluorane (1-2%), supplemented with oxygen (1-2 L). An abdominal midline incision was made and the inferior vena cava and the descending aorta isolated below the renal arteries. The recipient vessels were clamped with an atraumatic vascular clamp (ASSI, Westbury, NY) and opened with micro-scissors. The portal vein was anastomosed to the inferior vena cava and the donor aortic cuff was connected with an end-to-side anastomosis to the recipient aorta using 10.0 suture. The clamp was then opened to restore blood flow. Blood flow was confirmed using a blood pool agent (AngioSense-680, VisEn Medical, MA) and imaging as described previously.  

**Parabiosis:** The procedure, adapted from was conducted as previously described. Briefly, after shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from behind the ear to the tail of each mouse, and the subcutaneous fascia was bluntly dissected to create about $\frac{1}{2}$ cm of free skin. The scapulas were sutured using a mono-nylon 5.0 (Ethicon, Albuquerque, NM), and the dorsal and ventral skins were approximated by continuous suture. After an interval of three weeks, parabiosed mice were surgically separated by a reversal of the procedure. One group was splenectomized, as described above. Percent chimerism in the blood was defined for gated monocytes, neutrophils and Lin$^{1+}$ cells (mostly lymphocytes) as $\%$CD45.1 / ($\%$CD45.1 + $\%$CD45.2) in CD45.2 mice, and as $\%$CD45.2 / ($\%$CD45.2 + $\%$CD45.1) in CD45.1 mice.

**Adoptive transfer of GMP:** Donor CD45.1$^+$ or GFP$^+$ cells from the bone marrow were first enriched using anti-PE beads to deplete Lin$^{2+}$ cells (Miltenyi) and then sorted using a BD FACSaria II (BD Biosciences). Typically, purity of the sorted population was 99.5 %. $5 \times 10^4$ GMPs were injected into the tail vein of non-irradiated recipient mice. Anti-GM-CSF and IL-3 treatment: Mice were i.v. injected with blocking antibody against GM-CSF (MP1-22E9, eBioscience; 300 µg) twice daily for four consecutive days. Control animals received anti-mouse IgG2a (eBR2a, eBioscience; 300 µg). Monocyte reservoir depletion: Mice were i.v. injected with 250 µl clodronate loaded liposomes once. Clodronate was a gift from Roche and was incorporated into liposomes are described previously. Injection of oxLDL: Mice were i.v. injected once with Dil-oxLDL (Biomedical Technologies, Inc., Stoughton, MA; 100 µg). Endotoxin-induced peritonitis and peritoneal lavage: Mice were administered 10 µg of LPS.
(Sigma), unless otherwise stated, daily by i.p. injections in PBS over the course of 4 days. Controls received PBS alone. For select experiments, the peritoneal cavity was lavaged with 10 ml of PBS to retrieve infiltrated and resident leukocytes.

**Cells.** Peripheral blood for flow cytometric analysis was collected by cardiac puncture, using a 50 mM EDTA solution as anticoagulant. Erythrocytes were lysed using BD FACS Lysing Solution (BD Biosciences). Total white blood cell count was determined by preparing a 1:10 dilution of (undiluted) peripheral blood obtained from the orbital sinus using heparin-coated capillary tubes in RBC Lysis Buffer (BioLegend). After organ harvest, single cell suspensions were obtained as follows: for bone marrow, femur and tibia of one leg were flushed with PBS. Spleens were homogenized through a 40 µm-nylon mesh, after which erythrocyte lysis was performed on the spleens using RBC Lysis Buffer (BioLegend). For the flow cytometry experiments, the entire aorta was digested (from the root to the iliac bifurcation) according to a method previously published. The procedure involves perfusion of the aorta (20 ml PBS) prior to digestion. Aortic tissue was cut in small pieces and subjected to enzymatic digestion with 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 1 h at 37°C while shaking. Total viable cell numbers were obtained using Trypan Blue (Cellgro, Mediatech, Inc, VA). To determine total bone marrow cellularity, one femur was estimated to represent 5% of total marrow according to . For morphological assessment, cytopspins were prepared from 5 × 10⁴ cells and stained using HEMA 3 staining kit (Fisher Scientific). **Tissue colony forming cell assay:** To determine the number of myeloid colony-forming units in the steady state and in atherosclerosis, a single cell suspension was prepared from spleens and 1x10⁵ splenocytes were plated in triplicates in complete methylcellulose medium (MethoCult GF M3434, Stemcell Technologies) or methylcellulose medium lacking growth factors (MethoCult M3234, Stemcell Technologies) supplemented with 100ng recombinant murine GM-CSF and IL-3 according to the manufacturer’s instructions. In one set of experiments anti-GM-CSF and anti-IL-3 were added once to cultures at 10 µg/ml. Counts were performed after 8 days of culture. At least three independent samples per group were analyzed.

(BD Biosciences); anti-TER119-PE, Anti-TER119-FITC, anti-TER119- APC, TER119 (BD Biosciences); anti-NK1.1-PE, anti-NK1.1-FITC, anti-NK1.1-PerCP, PK136 (BD Biosciences); anti-CD11b-APC, M1/70 (BD Biosciences); anti-CD11b-PE (ED8) (Abcam); anti-CD11b-APC-Cy7 M1/70 (BD Biosciences); anti-F4/80-biotin, anti-F4/80-FITC, C1:A3-1 (BioLegend); anti-CD11c-biotin, anti-CD11c-FITC, anti-CD11c-APC, HL3 (BD Biosciences); anti-I-A<sup>b</sup>-biotin, anti-I-A<sup>b</sup>-FITC, AF6-120.1 (BD Biosciences); anti-F4/80-biotin, C1:A3-1 (BioLegend); anti-F4/80-PE-Cy7, BM8 (BioLegend); anti-Ly-6C-FITC, anti-Ly-6C-biotin, AL-21 (BD Biosciences); anti-Gr-1-PeCy7, RB6-8C5 (BD Biosciences); anti-CD45.2-FITC 104, anti-CD45.2-PerCP 104, anti-CD45.2-Alexa Fluor 700 (BD Biosciences); anti-CD45.1-biotin A20, anti-CD45.1-APC A20, anti-CD45.1-PerCP A20 (BD Biosciences); anti-CD34-FITC, anti-CD34-Alexa Fluor 700, RAM34 (BD Biosciences); anti-CD117-APC (BD Biosciences), anti-CD117-PE-Cy7 (eBioscience), 2B8; anti-Sca-1-PE-Cy7, anti-Sca-1-Alexa Fluor 700, D7 (eBioscience); anti-CD16/32-APC-Cy7, 2.4G2 (BD Biosciences); were used for flow cytometric analyses in this study. Streptavidin-PerCP was (BD Biosciences) were used to label biotinylated antibodies. Cathepsin activity was assessed using Prosense-680 (PerkinElmer). Cell cycle analysis was carried using FxCycle violet stain (Invitrogen). Apoptotic cells were identified as those cells falling within the subG<sub>1</sub> gate as previously described<sup>8</sup>. Contribution of newly-made cells to different cell populations was determined by in-vivo labeling with bromodeoxyuridine (BrdU). Mice received 1 mg of BrdU (BD Biosciences) by i.p. injection. Incorporation was measured using either FITC or APC-conjugated anti-BrdU antibodies according to the manufacturer’s instructions. Monocytes, macrophages/dendritic cells and neutrophils were identified as described previously.<sup>3</sup> Specifically, monocytes were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>−</sup> (Lin<sub>1</sub> = CD90/B220/CD49b/NK1.1/Ly-6G/Ter119) (F4/80/I-A<sup>b</sup>/CD11c)<sup>lo</sup>. Monocyte subsets were identified as either Ly-6C<sup>high</sup> or Ly-6C<sup>low</sup>. Macrophages/dendritic cells were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>−</sup> (F4/80/I-A<sup>b</sup>/CD11c)<sup>hi</sup> or on the basis of F4/80 expression only. Neutrophils were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>−</sup> (F4/80/I-A<sup>b</sup>/CD11c)<sup>lo</sup> Ly-6C<sup>int</sup>. Monocyte and macrophage/dendritic cell numbers were calculated as total cells multiplied by percent cells within the monocyte/macrophage gate. Hematopoietic stem cells were identified as Lin<sub>2</sub><sup>low</sup> (Lin<sub>2</sub> = CD90/B220/CD49b/Ter119/NK1.1/Gr-1/CD11b/CD11c) IL-7Ra<sup>−</sup> CD117<sup>+</sup> Sca-1<sup>+</sup>. Myeloid progenitors were identified as Lin<sub>2</sub><sup>low</sup> IL-7Ra<sup>−</sup> CD117<sup>+</sup> Sca-1<sup>−</sup>. Within this population, granulocyte/macrophage progenitors (GMP) were CD34<sup>+</sup> CD16/32<sup>+</sup>, common myeloid progenitors (CMP) were CD34<sup>+</sup> CD16/32<sup>+</sup> and megakaryocyte/erythroid progenitors (MEP) were CD34<sup>−</sup> CD16/32<sup>−</sup>. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v8.8.6 (Tree Star, Inc.). Cells were sorted on a BD FACSaria II (BD Biosciences).
Histology. Aortae and spleens were excised, embedded in O.C.T. compound (Sakura Finetek), and flash-frozen in isopentane and dry ice. Aortic roots were sectioned into 5 um slices, generating ~30-40 sections that spanned the entirety of the aortic root. For comparison of lesion size between the groups, the sections that captured the maximum lesion area were used. Sections adjacent to this were used for other histological staining. Immunofluorescence staining was carried out using the following primary antibodies: anti-CD11b: clone M1/70 (BD Biosciences), biotin anti-CD45.1: clone A20 (BioLegend), and anti-F4/80: clone CI:A3-1 (Abcam). In some cases, either streptavidin-Texas Red or streptavidin-fluorescein (GE Healthcare) were directly conjugated to primary antibodies. Biotinylated secondary antibodies were used when required. Specificity of staining was confirmed using relevant isotype controls. Cover slips were placed over specimens using mounting medium containing DAPI (Vector Laboratories) to identify cell nuclei. Samples were visualized using an epifluorescence microscope (Nikon Eclipse 80i, Nikon Instruments Inc.) equipped with a Cascade Model 512B camera (Roper Scientific). Immunohistochemical staining was used to detect GM-CSF (clone FL-144, Santa Cruz Biotechnology) and IL-3 (clone MP2-8F8, BioLegend) for spleen sections, and anti-CD11b (clone M1/70, BD Biosciences), anti-Mac3: clone M3/84 (BD Biosciences), anti-F4/80: clone CI:A3-1 (Abcam), and actin smooth muscle (NeoMarkers) for aortic roots. Specificity of staining was confirmed using relevant isotype controls. In order to block endogenous peroxidase activity, tissue sections were pre-treated with 0.3% hydrogen peroxide solution. Following application of appropriate biotinylated secondary antibodies, samples were developed using a Vectastain ABC kit (Vector Laboratories) and AEC substrate (DakoCytomation). All sections were counterstained with Harris Hematoxylin. Masson trichrome (Sigma) and Oil Red O (Sigma) staining were performed to visualize collagen and lipid content, respectively. Hematoxylin and eosin (H&E) staining was performed to assess overall tissue morphology. Captured images were digitized automatically using a Nanozoomer 2.0RS (Hamamatsu).

Intravital microscopy
Animal preparation: During isoflurane anesthesia, the peritoneal cavity was opened with a transverse incision in the disinfected abdominal wall. The gastro-splenic ligament was dissected and the spleen carefully exteriorized. Robust blood flow was observed in the splenic artery during the duration of each experiment and splenic perfusion was confirmed by inspection through fluorescence microscopy upon tail vein injection of an intravascular imaging agent. The
exteriorized spleen was completely submerged in temperature-controlled lactated Ringer’s solution. Temperature near the spleen was carefully monitored using an Omega HH12A thermometer with fine wire thermocouples (Omega Engineering Inc., Stamford, CT) and kept at 37°C. **Confocal Microscopy:** Images were collected with an intravital laser scanning microscope (IV100 vers. 1.2, Olympus Corporation, Tokyo, Japan) using an Olympus 4x UPlanSApo (NA 0.16), 10x UPlanFl (NA 0.3), 20x UPlanFl (NA 0.5) objective and the Olympus IV10-ASV 1.2 program. Samples were excited at 488 nm with an air-cooled argon laser (Melles Griot, Carlsbad, CA) for visualization of the GFP⁺ cells, at 561 nm with a solid state yellow laser (Melles Griot) for visualization of RFP, and at 748 nm with a red diode laser (Model FV10-LD748, Olympus Corporation, Tokyo, Japan) for visualization of the blood pool agent (AngioSense-750, VisEn Medical, MA). Light was collected using custom-built dichroic mirrors SDM-560 and SDM-640, and emission filters BA 505-550, 585-615 nm, and BA 770 nm IF (Olympus Corporation, Tokyo, Japan). The 488 nm and 750 nm channels were collected simultaneously. The 561 nm channel was collected line-sequentially to avoid bleed through between channels. Time-lapse recordings were made by collecting a z-stack of ten 512x512 pixels images at 10 μm interval every 30s. A cluster was defined as a group of cells (at least 5) in close proximity to one another (typically no more than 100 μm apart).
Supplemental Figure I

(a) Schematic for gating on monocytes, macrophages, neutrophils and other cells analyzed in this manuscript. (b) Enumeration of myeloid and lineage cells in spleens of C57BL/6 (wt) and apoE−/− mice consuming a high cholesterol diet (HCD) for 20 weeks. Data show that myeloid, but not lineage cells increase in number in apoE−/− mice consuming a HCD (means ± SEM, n =
6) *P< 0.0001. (c) Enumeration of monocytes and neutrophils in spleens of mice consuming HCD for up to 30 weeks. Data show that the number of monocytes and neutrophils increase over time in mice consuming HCD. (n= 51). Linear regression was performed on all data. (d) Enumeration of myeloid cells, monocytes and neutrophils in the spleens of LDLR−/−ApoB48−/− mice consuming Paigen’s diet for 30 weeks (means ± SEM, n = 5) *P< 0.0001. (e) Distribution of lymphoid and myeloid cells in the spleen, bone marrow and blood in animals that were splenectomized and received a spleen by transplantation and in control animals that were not splenectomized and did not receive a spleen by transplantation. Data show that spleen transplantation does not affect cell distribution in the various compartments. (means ± SEM, n = 8). (f) Pictures showing that the transplanted spleen is perfused. After transplant, Angiosense-680, a fluorescent blood pool agent, was injected and the spleen was imaged by near infrared imaging (NIR) and with a confocal microscope. A picture also shows the characteristic purplish red of the transplanted spleen.
Supplemental Figure II.

(a) Expression of pro-IL-1β on in vitro-unstimulated Lin−CD11b+ cells from the aorta of wt and apoE−/− mice consuming (HCD) for 20 weeks. Staining for the cytokine is shown as a contour plot and isotype staining is shown as a density plot in the back. Data are representative of at least three independent experiments and show that expression of pro-IL-1β increases in an inflammatory context.

(b) Isotype staining controls for pro-IL-1β antibody staining in aortic monocytes and macrophages.

(c) Proteolytic activity on monocytes retrieved from different organs. Data show that monocytes residing in the spleen and bone marrow (bm) have little proteolytic activity. This activity increases when monocytes accumulate in different sites.

(d)
Uptake of DiI-oxLDL by circulating Lin<sup>-</sup>CD11b<sup>+</sup> cells. Data show that CD45.2<sup>+</sup> (i.e., bone marrow) and CD45.1<sup>+</sup> (i.e. splenic) monocytes take up ox-LDL similarly. DiI-oxLDL was injected i.v. once. (e) Spleen transplantation for 10 days. Data show IF (left panels) on the aortic root with antibodies against CD45.1 (green) and F4/80 (red). DAPI depicts nuclei (blue). Yellow cells represent F480<sup>+</sup> cells of splenic origin. Oil red O staining (right panels) of the same aortic root tissue section reveals co-localization of lipid accumulation and lesional cells of splenic origin. (f) H&E staining of aortic root lesions of spleen-containing and asplenic apoE<sup>−/−</sup> HCD mice. (g) Immunohistochemical staining for macrophage/monocyte markers in aortic root sections from mice in (f). Depicted is representative staining for Mac3, CD11b, and F4/80. (h) Masson trichrome staining of aortic roots from representative control and splenectomized mice. (i) Actin smooth muscle staining.

**Supplemental Figure III**

(a) Enumeration of Lin<sub>2</sub>-Sca<sub>c</sub>-kit<sup>+</sup> (LSK), common myeloid progenitor (CLP), granulocyte-macrophage precursors (GMP), and common lymphoid progenitor cells (CLP) in spleens of C57BL/6 (wt) and apoE<sup>−/−</sup> mice consuming a high cholesterol diet (HCD) for 8 weeks. Data show that myeloid progenitor populations, but not lineage progenitor cells increase in number in apoE<sup>−/−</sup> mice consuming a HCD. (b) Enumeration of GMP in the spleens of LDLR<sup>−/−</sup>ApoB48<sup>−/−</sup> mice consuming Paigen’s diet. (means ± SEM, n = 5-8) *P< 0.05. (c) Representative post-sort
flow cytometric analysis of GMPs that were used for adoptive transfer. Overall purity of GMPs within the living population was 99.5%. This is a quality control experiment showing that the GFP+ GMP that are injected are sorted to high purity. (d) Adoptive transfer of GFP+ GMP to wt and apoE−/− HCD mice. Data show GFP cells in spleens 3 days after transfer. Increased percentage of GFP+ progenitors and myeloid cells was observed in spleens of apoE−/− HCD mice.

Supplemental Figure IV.
(a) Isotype staining for GM-CSF and IL-3 on spleens from apoE−/− mice consuming HCD for 20 weeks. (b) Flow cytometry intracellular staining for GM-CSF, IL-3 and CD45 in spleens of apoE−/− mice consuming HCD for 20 weeks. Representative plots of at least three independent experiments are shown. Isotype controls were used to determine positive staining. (c) Effect of IL-3 and GM-CSF neutralization on the endogenous GMP and CD11b+Gr1+ repertoires in the bone marrow (means ± SEM, n = 5).
Supplemental Figure V.

(a) Picture of spleens retrieved from animals in the steady state and 4 days after daily injection of lipopolysaccharide (LPS). (b) Enumeration of neutrophils and monocytes in the blood and spleen in the steady state and in response to LPS. Numbers in gray show the fold increase for a given population (means ± SEM, n = 3-9).*P< 0.05. (c) H&E staining and IF on CD11b on spleen sections from naïve mice or mice that received LPS. Data show that in response to LPS...
the red pulp increases in size and this increase is associated with a higher number of CD11b+ cells. Clusters can be detected in the red pulp of the LPS-treated mice. (d) Representative flow cytometric plots and enumeration depict the phenotype and fold increase of granulocyte macrophage progenitors (GMP) in spleens of C57BL/6 mice in the steady state and 4 days after daily injection of lipopolysaccharide (LPS). Bars show the fold increase over steady state in spleen and bone marrow (means ± SEM, n = 3 to 5). *P< 0.05. (e) DAPI staining shows the percentage of GMPs that are in the S/G2 phase of the cell cycle. A representative plot of at least three independent experiments is shown. (f) Clonal efficiency and colony formation. Single progenitors were sorted into 96-well plates and scored for colonies after 8 days. Percentages of positive wells are shown in parentheses. The data show that GMPs isolated from the spleen and the bone marrow have a similar clonal efficiency. The graph depicts number of CFU-GM in spleen in the steady state and after LPS (means ± SEM, n = 3 to 5). *P< 0.05. (g) Intravital microscopy pictograms of the spleen red pulp depict clusters of GFP+ cells adoptively transferred i.v. 3, 8 and 12 days earlier into inflammatory (LPS-injected) mice. Vasculature is shown in red and the scale is depicted with white bars. The large panels show clusters on day 8 (h) Enumeration of cluster number and cluster size in the subcapsular red pulp 5, 8 and 12 days after transfer of GFP+ GMPs (means ± SEM, n = 4 to 9). (i) Phenotype of injected GFP+ cells in the spleen on day 8. Data show that on day 8 after injection of GFP+ GMP all the cells detected in the spleen are neutrophils and monocytes and not GMPs. (j) Cartoon depicts experimental design for the co-injection of equal numbers of GFP+ GMPs and RFP+ GMPs into C57BL/6 mice injected with LPS. (k) Green and red clusters in the subcapsular red pulp 5 days after injection of equal numbers of GFP+ GMPs and RFP+ GMPs. Vasculature is shown in blue and the scale is depicted by a white bar. Data are representative of at least three independent experiments. Data show that GMPs clonally expand in the spleen. If they did not clonally expand, a mixture of green and red cells would be expected. However, the clusters are either exclusively green or exclusively red and never mixed, thus showing that monocytes and neutrophils are made in the spleen.

References (to Expanded Methods)


