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Citation

Robbins, Clinton S., Aleksey Chudnovskiy, Philipp J. Rauch, Jose-Luiz Figueiredo, Yoshiko Iwamoto, Rostic Gorbatov, Martin Etzrodt, et al. 2012. "Extramedullary Hematopoiesis Generates Ly-6C High Monocytes That Infiltrate Atherosclerotic Lesions." Circulation 125 (2): 364–74. https://doi.org/10.1161/circulationaha.111.061986.

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Circulation. Author manuscript; available in PMC 2013 January 17.

Published in final edited form as:
Circulation. 2012 January 17; 125(2): 364–374. doi:10.1161/CIRCULATIONAHA.111.061986.

Extramedullary Hematopoiesis Generates Ly-6C^{high} Monocytes that Infiltrate Atherosclerotic Lesions

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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^{high} monocytes are inflammatory, accumulate in lesions preferentially, and differentiate. Here we hypothesized that the bone marrow outsources the production of Ly-6C^{high} 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^{high} monocytes. Monocytes born in such extramedullary niches intravasate, circulate, and accumulate abundantly in atheromata. Upon lesional infiltration, Ly-6C^{high} 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.

Keywords

Atherosclerosis; Imaging; Immune System; Immunology; Macrophage

Monocytes are myeloid leukocytes that circulate in the blood and patrol the vascular endothelium.[1–4] 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

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Conflict of Interest Disclosures: None

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the periphery, and differentiate.[5, 6] 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.[7–10] Among leukocytes, monocytes are essential to the disease's development and exacerbation.[4, 11, 12] 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.[12, 13] Ly-6C^{high} monocytes are also believed to convert to Ly-6C^{low} monocytes[14, 15], but this conversion is compromised during atherosclerosis.[12] 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[16–18], becomes monocytopoietic during atherosclerosis.

Methods

Animals

C57BL/6J (wt), B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1^{+}), C57BL/6-Tg(UBC-GFP)30Scha/J (GFP^{+}), B6.Cg-Tg(ACTB-mRFP1)1F1Hadj/J (RFP^{+}), ApoE$^{-/-}$ mice (B6.129P2-Apoe^{tm1Unc}), LDLR$^{-/-}$ ApoB48$^{-/-}$ (B6;129S-Apob^{tm2Sgy} Ldlr^{tm1Her}/J) 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.

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.

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 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. 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.

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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^{high} monocytes to the growing atheroma

We have previously shown that the spleen contains a monocyte reservoir that mobilizes in response to acute injury[17], 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$^{-/-}$ mice. Immunoflourescent staining, which provides information on spatial distribution, showed expansion of CD11b^{+} cells throughout the red pulp of ApoE$^{-/-}$ mice (Figure 1A), indicating enlargement of this reservoir. Enumeration of splenic leukocytes in wild type C57BL/6 mice and ApoE$^{-/-}$ mice consuming a diet high in fat and cholesterol (HCD) revealed dramatic differences between the groups: ApoE$^{-/-}$ 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[12]. 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$^{-/-}$ ApoB48$^{-/-}$ 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$^{-/-}$ mice to recently-splenectomized 30 week old CD45.2 ApoE$^{-/-}$ mice that had consumed a HCD for 20 weeks. The transplantation procedure anastomoses splenic and recipient vessels, preserves blood flow and organ integrity [17], 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$^{-/-}$ 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$^{-/-}$ mice consuming a chow diet (chow) because they contained small spleens and relatively small myeloid reservoirs (Figure 1B), and spleens from CD45.1 ApoE$^{-/-}$ mice consuming HCD that had their reservoirs depleted with clodronate liposomes (Figure 1B).

Transplantation of CD45.1 ApoE$^{-/-}$ mice consuming HCD for 20 weeks led to a large accumulation of spleen-derived monocytes, but not macrophages, in the recipient blood ($4.7 \times 10^5 \pm 2 \times 10^5$ 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^{+}Lin$_1^{-}$, 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^{+} cells in the recipient aorta consisting of F4/80^{low} monocytes and F4/80^{high} macrophages (Figure 1D), thus indicating that spleen-experienced F4/80^{low}

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monocytes accumulated in lesions and matured to F4/80^{high} 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^{high} whereas monocytes arriving from all other sources (i.e., CD45.2) contained a mixture of Ly-6C^{high} andLy-6C^{low} 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$^{-/-}$ 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^{high} 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 remodels the extracellular matrix, and lipid uptake yields foam cells.[7] 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.[19] 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 cysteinyl cathepsin activity in vivo.[20, 21] 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.[22] Regardless of origin, monocytes and

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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.[23]. In animal models, splenectomized mice [24, 25] as well as hamsters depleted of monocytes with clodronate liposomes, develop larger lesions.[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 accelular 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 IIi). 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[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$^{-/-}$ HCD mice formed many more granulocyte/macrophage colonies than wt controls (Figure 3A), indicating clonal myeloid-cell proliferation. Compared to wt controls, ApoE$^{-/-}$ HCD mice contained numerous HSPC, including LSK (Lin_2^- Sca-1$^+$ c-kit$^+$) 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[4] (Figure 3B and online-only Data Supplement Figure IIIa). Common lymphoid progenitors (CLP) did not increase in ApoE$^{-/-}$ 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) [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$^{-/-}$ ApoB48$^{-/-}$ 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

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ApoE$^{-/-}$ HCD or wt mice and enumerated GFP^{+} cells 5 days later. Considerably more GFP^{+} cells accumulated in the spleens of ApoE$^{-/-}$ HCD mice than the controls (Figure 3D, E). Among these cells we detected progenitors and differentiated CD11b^{+}Gr1^{+} monocytes and neutrophils (online-only Data Supplement Figure IIId), which indicates GMP expansion. The number of GFP^{+} cells accumulating in the bone marrow, however, was similar in wt and ApoE$^{-/-}$ 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[11, 28], and adoptive transfer of 5×10^6 sorted monocytes by a procedure that allows their retrieval 1 day later[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^{+} ApoE$^{-/-}$ 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.[17] 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.

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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[29], but their role in atherosclerosis is poorly understood.[30–32] Recent observations in experimental atherosclerosis that HSPCs increase the common receptor subunit for GM-CSF and IL-3[33], 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 G_2 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)-

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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-$6C^{high}$ monocytes ($CD11b^+$ $Gr1^+$ 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 $CD11b^+$ $Gr1^+$ 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 [34]. GFP GMPs and RFPGMPs 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[35], whereas the second argued that, in the steady state, the bone marrow is the exclusive monocyte production site.[36] Multiple studies since have enriched our understanding of these processes. Extramedullary hematopoiesis occurs in development and in a number of genetic and myeloproliferative conditions.[37–41] 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.[2] 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 comparmentalized in different organs. In atherosclerosis, our data imply, the bone marrow outsources the production of circulating leukocytes.

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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[23], yet it is capable of cell retention through a myriad of adhesive ligands.[42, 43] The organ can accomodate 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.[17] 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 constitue the organ.[44] The observations made here in response to hypercholesterolemia or endotoxin indicate a role for the spleen in supplying Ly-6C^{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[24] can be spared.

That extramedullary generation of Ly-6C^{high} monocytes 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.[5, 45] HSPC differentiation in extramedullary sites may be essential to the replenishment of dendritic cells and macrophages.[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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Michael T. Waring and Adam T. Chicoine for sorting cells (Ragon Institute Imaging Core at Massachusetts General Hospital). The authors thank Melissa Greene for secretarial assistance.

Funding Sources: This work was supported in part by NIH grants 1R01 HL095612 (F.K.S.), U01 HL080731, P50 CA86355, U24 CA092782 and P01-A154904 (to R.W.), 2R01 HL069948 (to M.J.M.-K). C.S.R. was supported by

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the American Heart Association (AHA) postdoctoral fellowship and the Massachusetts General Hospital ECOR Postdoctoral Award. P.J.R. was supported by the Boehringer Ingelheim Fonds. G.F.W. was supported by the German Research Foundation.

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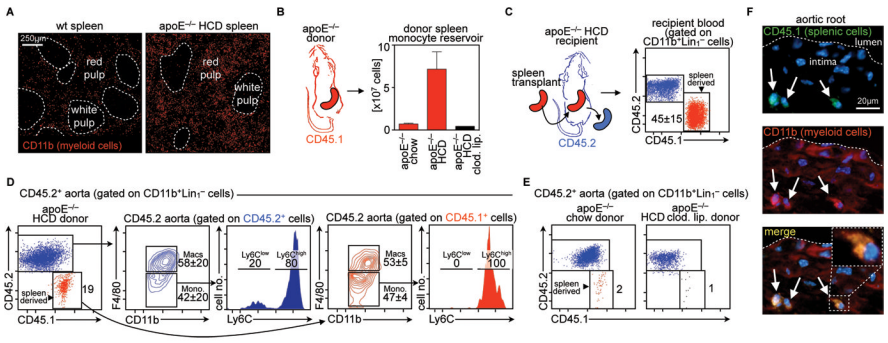

Figure 1.

Splenic myeloid cells infiltrate atherosclerotic lesions. **A.** Immunofluorescence (IF) of CD11b cells (red) in spleens of C57BL/6 (wt) and apoE$^{-/-}$ 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$^{-/-}$ mice consuming a chow diet for 20 weeks, apoE$^{-/-}$ mice consuming HCD 20 weeks, and apoE$^{-/-}$ mice consuming HCD for 20 weeks and then injected with clodronate liposomes 1 day prior (n = 2–10). **C.** Presence of CD45.1^{+} cells in the blood of CD45.2^{+} mice that received CD45.1^{+} spleens by transplantation. **D.** Spleen transplantation from CD45.1 apoE$^{-/-}$ donors consuming HCD to CD45.2 apoE$^{-/-}$ recipients. Data show direct accumulation and differentiation of splenic Ly-6C^{high} monocytes in aortic lesions in 1 day. One of 11 representative experiments is shown. **E.** Spleen transplantation from CD45.1 apoE$^{-/-}$ donors consuming chow and from CD45.1 apoE$^{-/-}$ 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^{+} cells of splenic origin. For all flow cytometric plots the ticks represent 0, 10^2, 10^3, 10^4, 10^5 mean fluorescence units (MFI), except for axes labled “cell no.” or “SSC” where the ticks represent 0, 50K, 100K, 150K, 200K and 250K MFI.

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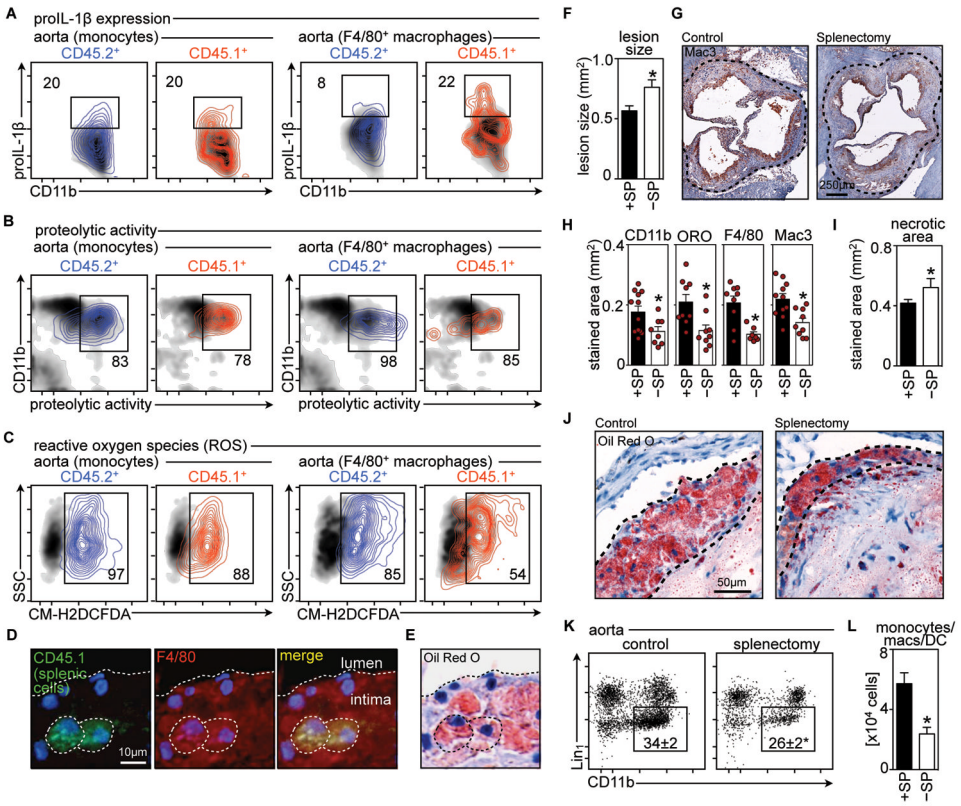

Figure 2.

Splenic cells are inflammatory and shape lesional evolution. **A.** Spleen transplantation from CD45.1^{+} apoE$^{-/-}$ to CD45.2^{+} apoE$^{-/-}$ 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.

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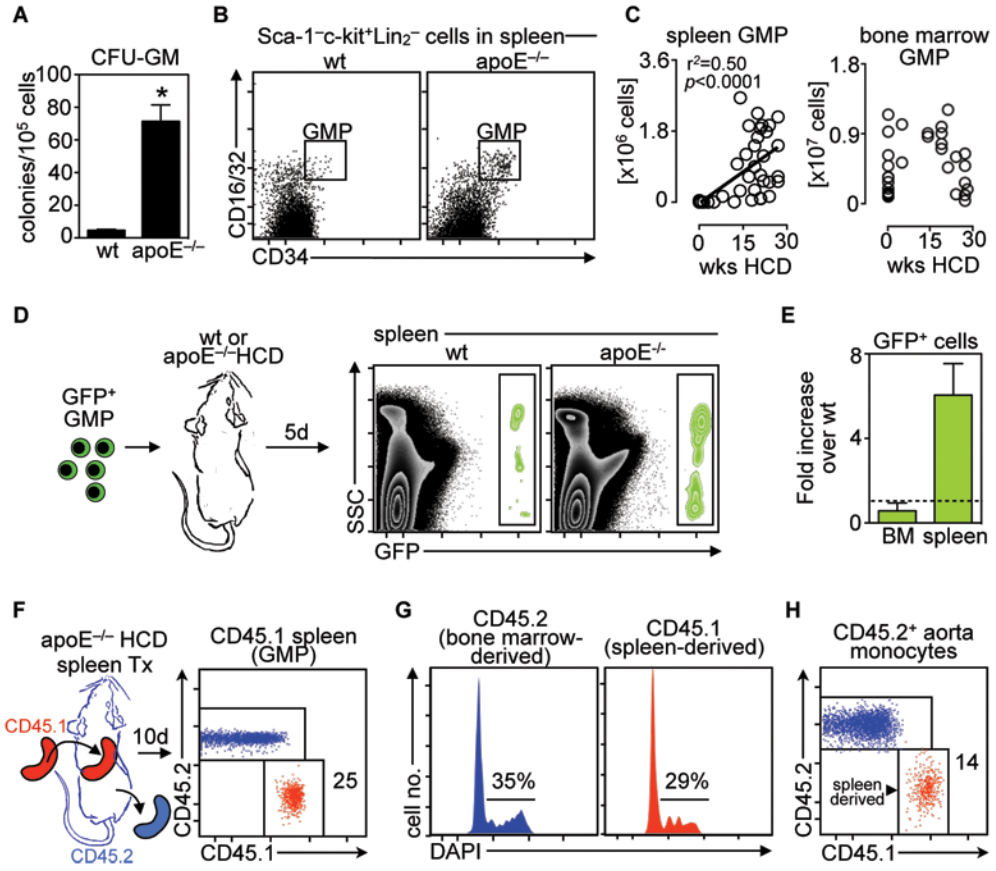

Figure 3.

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$^{-/-}$ HCD mice (means ± SEM, n = 4). *P< 0.05. **B.** Phenotypic analysis of granulocyte and macrophage progenitors (GMP) in spleens of wt and apoE$^{-/-}$ HCD mice. **C,** Enumeration of GMP in spleens and bone marrow of apoE$^{-/-}$ mice fed HCD for up to 30 weeks. Linear regression was performed. **D.** Adoptive transfer of GFP^{+} GMP to wt and apoE$^{-/-}$ 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$^{-/-}$ HCD mice of adoptively transferred GFP^{+} cells in the bone marrow (BM) and spleen (data shown are pooled from two independent experiments). **F.** CD45.1^{+} spleens from apoE$^{-/-}$ HCD mice were transplanted to asplenic CD45.2^{+} apoE$^{-/-}$ HCD mice for 10 days. Data show chimerism of GMP in spleens 10 days after transplantation. **G.** Cell cycle analysis of CD45.1^{+}and CD45.2^{+} GMP in transplanted spleens shown in G. Numbers indicate percentage of cells in S/G_2/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.

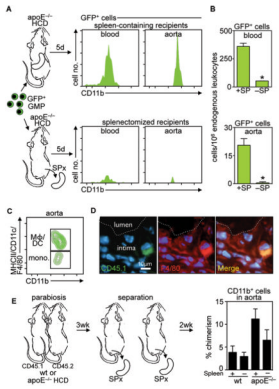

Figure 4.

Splenic monocytopoiesis gives rise to lesion-infiltrating monocytes. **A.** Adoptive transfer of GFP^+ GMP to $apoE^{-/-}$ HCD mice with or without their spleen. Data show GFP^+ 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^+GMPs into lesional macrophages. A representative contour plot shows that GFP^+ cells that were injected as GMP have accumulated in lesions as monocytes and matured to macrophages. **D.** CD45.1 GMP were adoptively transferred to $apoE^{-/-}$ 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 $apoE^{-/-}$ HCD mice were joined in parabiosis for 3 weeks, separated, splenectomized (or not) and assessed for chimerism 2 weeks later. Data show chimerism for $CD11b^+$ cells in the aorta (means ± SEM, n = 4).

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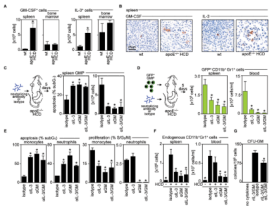

Figure 5.

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$^{-/-}$ 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$^{-/-}$ HCD mice. **C.** Effect of IL-3 and GM-CSF neutralization on endogenous splenic GMP. Data show percent of cells in subG$_1$ 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$^+$ GMP pulse chase. Data show GFP$^+$ CD11b$^+$Gr1$^+$ 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$^+$Gr1$^+$ 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).

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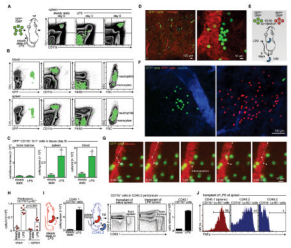

Figure 6.

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.

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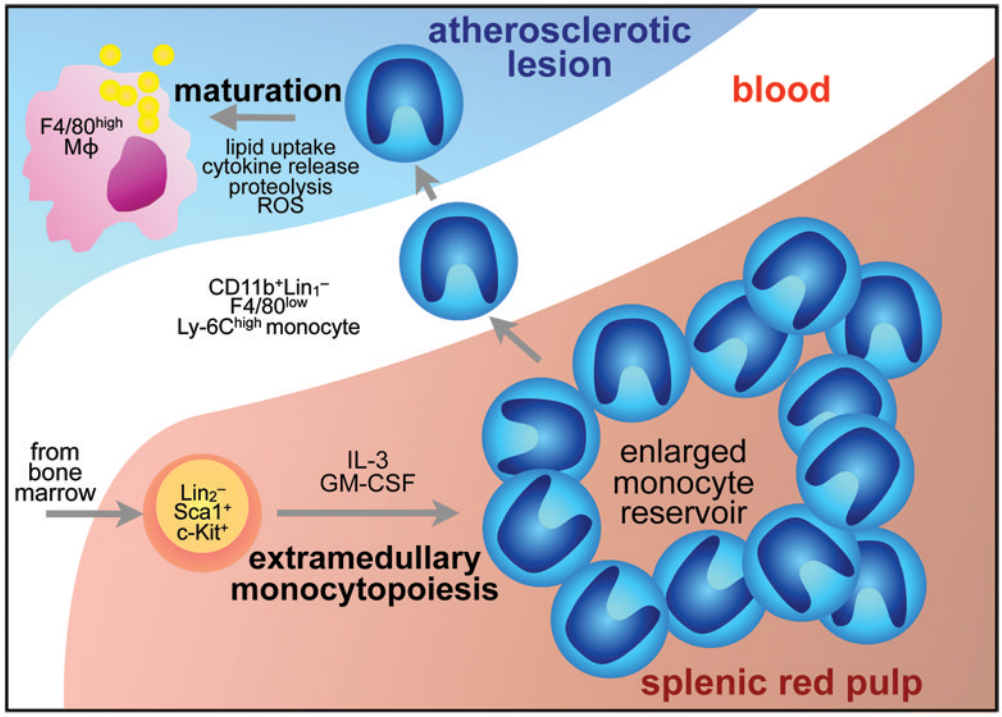

Figure 7.

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.