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

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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 progressively relocate from the bone marrow to the splenic red pulp, where they encounter granulocyte macrophage colony-stimulating factor and interleukin-3, clonally expand, and differentiate to Ly-6C^{high} monocytes. Monocytes born in such extramedullary niches intravasate, circulate, and accumulate abundantly in atheromata. On 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 hematopoietic function of the bone marrow by producing circulating inflammatory cells that infiltrate atherosclerotic lesions. (***Circulation*. 2012;125:364-374.**)

Key Words: atherosclerosis ■ imaging ■ immune system ■ immunology ■ macrophages

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 (HSPCs), which are developmentally upstream, readily mobilize from their bone marrow niches, accumulate in the periphery, and differentiate.[5,6] Although this phenomenon of extramedullary hematopoiesis is known to give rise to erythrocytes, platelets, granulocytes, and dendritic cells, it remains unknown whether HSPCs can yield monocytes outside the bone marrow. Likewise, the mechanisms that govern HSPC proliferation and differentiation and the eventual fate of the various progeny remain elusive.

Clinical Perspective on p 374

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 development and exacerbation of the disease.[4,11,12] On lesional accumulation and as a consequence of ingesting lipids abundantly, monocyte-derived macrophages become foam cells, the key culprits of atherosclerotic complications. Of the 2 recognized murine monocyte subsets, Ly-6C^{high} monocytes have been shown to accumulate preferentially in growing lesions and to 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 contribute 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

Received August 22, 2011; accepted November 22, 2011.

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Guest Editor for this article was Donald D. Heistad, MD.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.111.061986/-/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.111.061986**

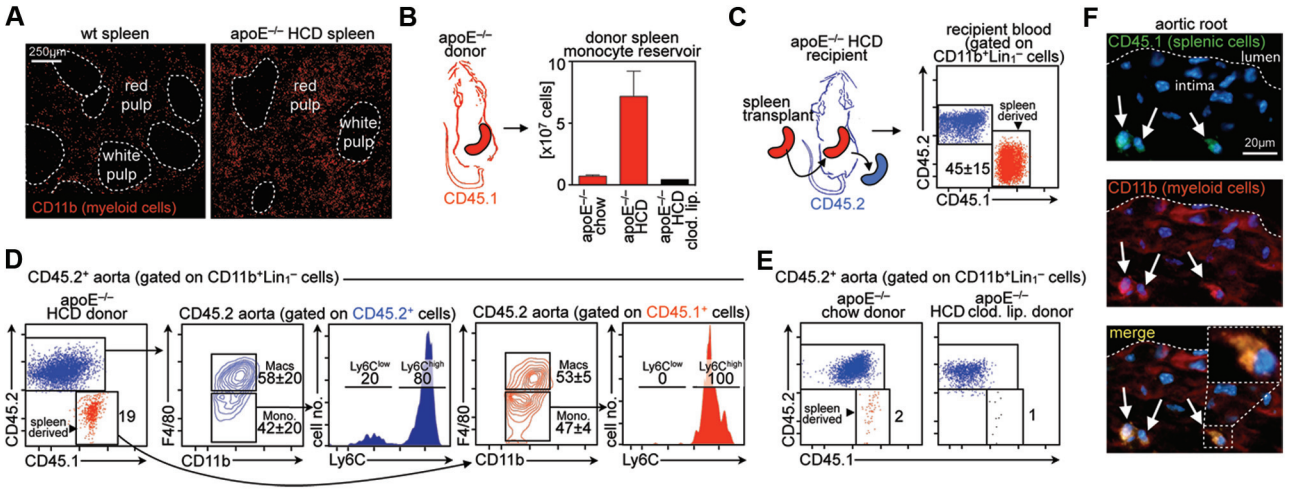

Figure 1. Splenic myeloid cells infiltrate atherosclerotic lesions. **A**, Immunofluorescence (IF) of CD11b cells (red) in spleens of C57BL/6 (wt) and apolipoprotein E–deficient (ApoE$^{-/-}$) mice consuming a high-cholesterol diet (HCD) for 20 weeks. Data show that the red pulp myeloid component enlarges 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 an HCD 20 weeks, and ApoE$^{-/-}$ mice consuming an HCD for 20 weeks and then injected with clodronate liposomes 1 day earlier (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 an 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 an 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) and CD11b (red) and the merge of the 2 (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 fluorescence units, except for axes labeled "cell no." or "SSC," for which the ticks represent 0, 50 000, 100 000, 150 000, 200 000, and 250 000 fluorescence units.

contains a reservoir of undifferentiated monocytes in the steady state,[16–18] becomes monocytopoietic during atherosclerosis.

Methods

Animals

C57BL/6J (wild-type [wt]), B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (CD45.1^{+}), C57BL/6-Tg(UBC-GFP)30Scha/J (green fluorescent protein–positive [GFP^{+}]), B6.Cg-Tg(ACTB-mRFP1)1F1Hadj/J (red fluorescent protein–positive [RFP^{+}]), apolipoprotein E–deficient (ApoE$^{-/-}$) mice (B6.129P2-Apoe^{tm1Unc}), and low-density lipoprotein receptor–deficient (LDLR$^{-/-}$)ApoB48$^{-/-}$ (B6;129S-Apob^{tm2Sgy} Ldlr^{tm1Her}/J) male and female mice were purchased from The Jackson Laboratories. All protocols were approved by the Animal Review Committee at Massachusetts General Hospital. More details are given 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 the Experimental Procedures section in the online-only Data Supplement. Mice were injected intravenously with blocking antibodies, with clodronate liposomes, or with oxidized LDL, as described in the 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 version 8.8.6 (Tree Star, Inc). Cells were sorted on a BD FACSAria II (BD Biosciences).

Histology

Aortas and spleens were excised, embedded in optimal-cutting-temperature compound (Sakura Finetek), and flash-frozen in isopentane and dry ice. Frozen 5-μm-thick sections were used in all staining protocols. Immunofluorescence staining was carried out with the 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 given in the online-only Data Supplement.

Statistics

Results are expressed as mean±SEM. Statistical tests included unpaired, 2-tailed Student t test with the Welch correction for unequal variances and 1-way ANOVA followed by the Bonferroni comparison test. Values of $P \leq 0.05$ 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. Immunofluorescent 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 wt 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 (Figure Ia and Ib in the online-only

Data Supplement), a finding that complements previous work.[12] Within the myeloid compartment of the spleen, both monocyte and neutrophil numbers increased (Figure Ic in the online-only Data Supplement). We also detected an expansion of monocytes and neutrophils in the spleens of LDLR$^{-/-}$ApoB48$^{-/-}$ mice consuming a Paigen diet, indicating that the phenomenon is ApoE independent (Figure Id in the online-only Data Supplement).

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 an 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 (Figure Ie in the online-only Data Supplement). The transplanted spleens resembled endogenous spleens in size and cellularity, had the characteristic purplish-red color, and were well perfused (Figure If in the online-only Data Supplement). Spleens of CD45.1 ApoE$^{-/-}$ mice consuming an 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 an HCD that had their reservoirs depleted with clodronate liposomes (Figure 1B).

Transplantation of CD45.1 ApoE$^{-/-}$ mice consuming an HCD for 20 weeks led to a large accumulation of spleen-derived monocytes, but not macrophages, in the recipient blood ($4.7\times10^5\pm2\times10^5$ 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 Ia in the online-only Data Supplement), 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} 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 reside in the aorta before 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 (ie, CD45.1) were Ly-6C^{high}, whereas monocytes arriving from all other sources (ie, CD45.2) contained a mixture of Ly-6C^{high} and Ly-6C^{low} monocytes. Transplantation of spleens from either of the 2 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 with age- and diet-matched controls that did not receive a spleen by transplantation (Figure Ig in the online-only Data Supplement), 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–Interleukin-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 with 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 call these spleen-experienced monocytes and their descendent macrophages "splenic" and 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 interleukin (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 with wt controls (Figure IIa and IIb in the online-only Data Supplement), reflecting the higher inflammatory burden of these animals. Spleen transplantation revealed similar pro–IL-1β expression among splenic (CD45.1$^+$) and medullary (CD45.2$^+$) monocytes, suggesting that the inflammatory capacity of splenic monocytes is comparable to that of their counterparts (Figure 2A). Splenic (CD45.1$^+$) aortic macrophages remained pro–IL-1β$^+$ at proportions similar to those of 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 with lesional monocytes and macrophages accumulating from the bone marrow (CD45.2$^+$) revealed a similar proportion (83% versus 78%; 98% versus 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 (Figure IIc in the online-only Data Supplement). To measure cellular redox states, we used an intracellular probe that measures oxidative stress.[22] Regardless of origin, monocytes and macrophages expressed reactive oxy-

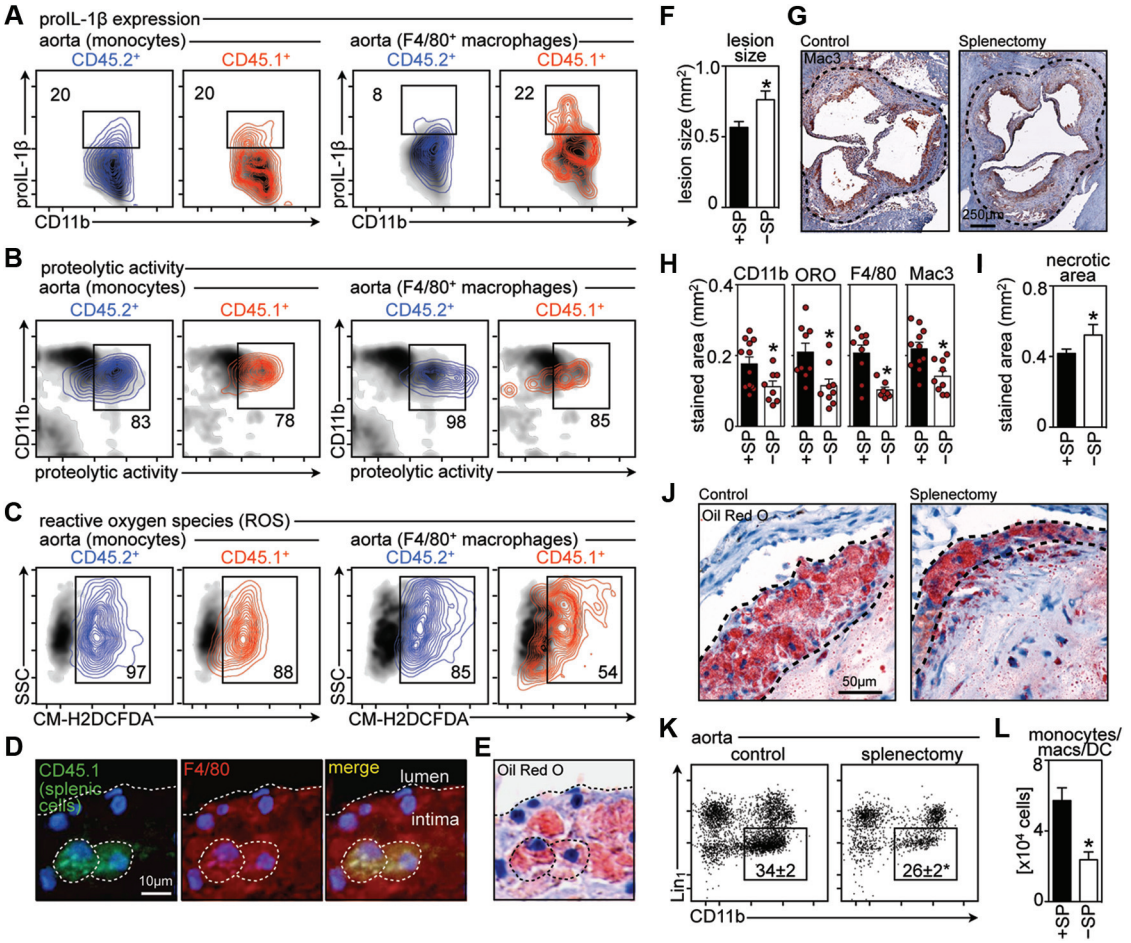

Figure 2. Splenic cells are inflammatory and shape lesional evolution. **A**, Spleen transplantation from CD45.1$^+$ apolipoprotein E–deficient (ApoE$^{-/-}$) to CD45.2$^+$ ApoE$^{-/-}$ mice. Expression of pro–interleukin (IL)-1β on monocytes and macrophages directly ex vivo (ie, unstimulated). Contour plots show pro–IL-1β expression gated on lesional monocytes and macrophages of splenic (CD45.1$^+$, red) or other source (ie, 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 (ie, bone marrow; CD45.2$^+$, blue) origin. The control density plots (black) are gated on all leukocytes. **C**, Contour plots show the presence of reactive oxygen species on lesional monocytes and macrophages of splenic (CD45.1$^+$, red) or other source (ie, 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** through **C** of at least 2 independent experiments are shown. **D**, Spleen transplantation for 10 days. Data show immunofluorescence on the aortic root with antibodies against CD45.1 (splenic cells, green), F4/80 (macrophages, red), and their merge (yellow). DAPI depicts nuclei (blue). Arrows point to F4/80$^+$ cells of splenic origin. **E**, Oil Red O (ORO) staining of the same section as in **D** shows colocalization of ORO with spleen-derived macrophages. **F**, Splenectomy of ApoE$^{-/-}$ high-cholesterol diet (HCD) mice for 12 weeks. Data show enumeration of total lesion size with hematoxylin and eosin. **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/dendritic cells enumerated by flow cytometry (mean±SEM; n=5–11) *P<0.05.

gen 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-oxidized LDL to the same extent (Figure IId in the online-only Data Supplement) and aortic atheromata contained large myeloid cells of splenic origin that took up Oil Red O (Figure 2D and 2E and Figure IIe in the online-only Data Supplement), 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] and 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 hematoxylin and eosin staining (Figure IIf in the online-only Data Supplement). 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

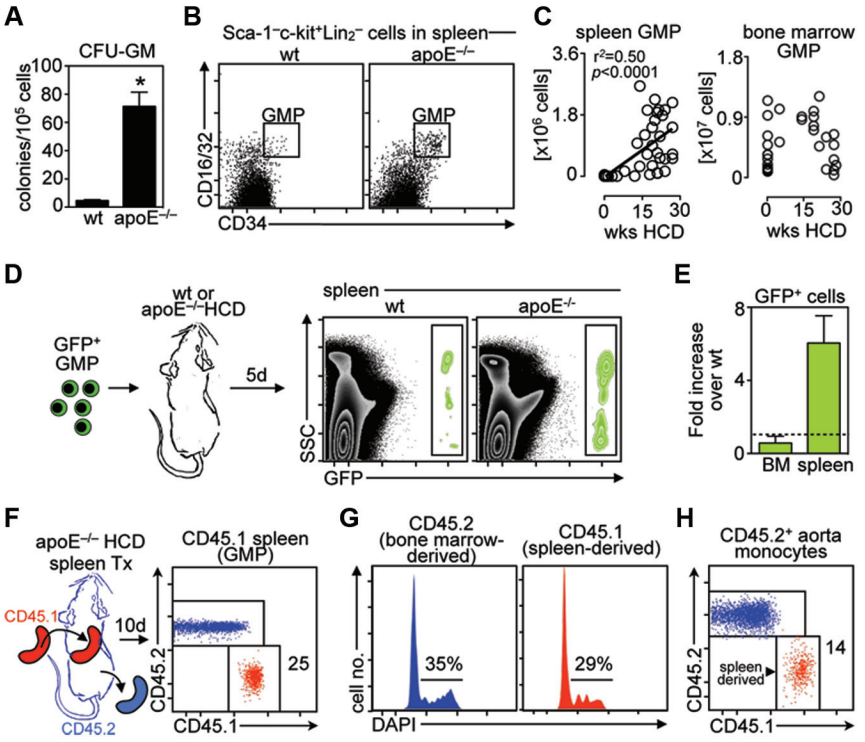

Figure 3. The spleen contains proliferating myeloid cell progenitors that give rise to their progeny in vivo. **A**, Colony-forming units–granulocytes and macrophages (CFU-GM) show colony formation in spleens of wild-type (wt) and apolipoprotein E–deficient ($ApoE^{-/-}$) high-cholesterol diet (HCD) mice (mean±SEM; n=4). *$P<0.05$. **B**, Phenotypic analysis of granulocyte and macrophage progenitors (GMPs) in spleens of wt and $ApoE^{-/-}$ HCD mice. **C**, Enumeration of GMP in spleens and bone marrow of $ApoE^{-/-}$ mice fed an HCD for up to 30 weeks. Linear regression was performed. **D**, Adoptive transfer of green fluorescent protein–positive (GFP^+) GMPs 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 2 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 GMPs in spleens 10 days after transplantation. **G**, Cell cycle analysis of $CD45.1^+$ and $CD45.2^+$ GMP in transplanted spleens shown in **F**. 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**. Representatives of 2 independent experiments are shown.

splenectomized mice contained fewer monocyte/macrophages (Figure 2G and 2H and Figure IIg in the online-only Data Supplement) but had larger acellular areas (Figure 2I). Accordingly, cellular Oil Red O areas were smaller in splenectomized mice (Figure 2H and 2J). We detected no differences in collagen content, as measured by Masson trichrome (Figure IIh in the online-only Data Supplement) and no differences in smooth muscle cell content (Figure IIi in the online-only Data Supplement). Flow cytometry of digested aortas confirmed that lesions of splenectomized mice contained fewer monocytes/macrophages (Figure 2K and 2L).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 their 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 with wt controls, $ApoE^{-/-}$ HCD mice contained numerous HSPCs, including LSK (Lin_2^- $Sca\text{-}1^+$ $c\text{-}kit^+$) cells and common myeloid progenitors, as well as granulocyte and macrophage progenitors (GMPs), which are the most committed progenitors known to give rise to monocytes and neutrophils[4] (Figure 3B and Figure IIIa in the online-only Data Supplement). Common lymphoid progenitors did not increase in $ApoE^{-/-}$ HCD mice, indicating a preference toward the myeloid lineage in the spleen (Figure IIIa in the online-only Data Supplement). GMP enumeration over 30 weeks of diet showed continued growth in the spleen. The GMP population in the bone marrow also grew, but less markedly and transiently (Figure 3C). Indeed, in many older animals, the GMP population in the spleen exceeded that of the bone marrow. Notably, although the spleen also contained macrophage and dendritic cell progenitors,[27] the aorta and para-aortic lymph nodes were virtually devoid of HSPCs (data not shown). In keeping with the expanded reservoir, the spleens of $LDLR^{-/-}ApoB48^{-/-}$ mice also contained higher numbers of GMPs (Figure IIIb in the online-only Data Supplement). These findings demonstrate that the spleens of atherosclerotic mice contain the requisite myelopoietic cells.

To test for monocytopoiesis in vivo, we injected 1×10^5 highly purified GFP-expressing GMP (Figure IIIc in the online-only Data Supplement) into either 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 in the controls (Figure 3D and 3E). Among these cells, we detected progenitors and differentiated CD11b$^+$Gr1$^+$ monocytes and neutrophils (Figure IIId in the online-only Data Supplement), 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 intravenously).

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 to 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, that 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 naïve 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.

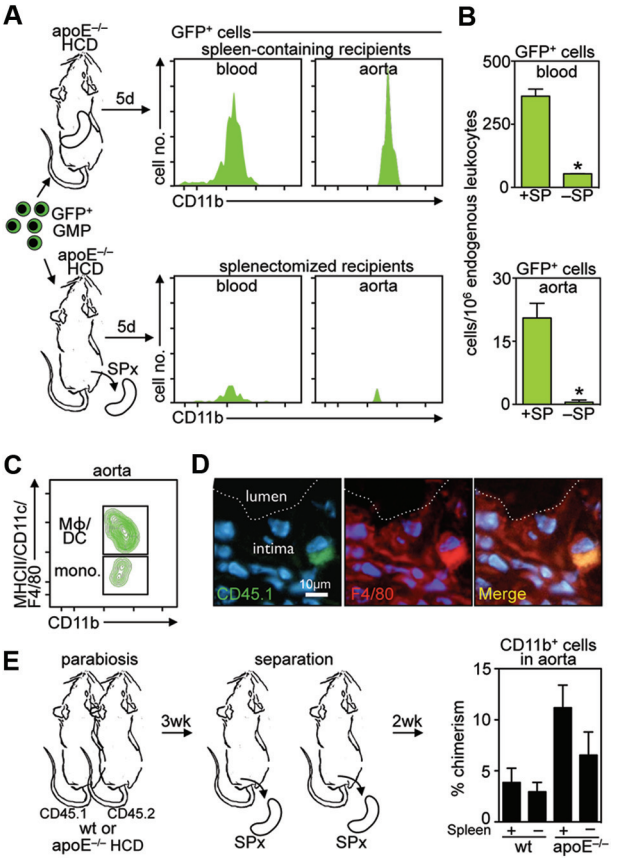

Figure 4. Splenic monocytopoiesis gives rise to lesion-infiltrating monocytes. **A**, Adoptive transfer of green fluorescent protein–positive (GFP$^+$) granulocyte and macrophage progenitors (GMPs) to apolipoprotein E–deficient (ApoE$^{-/-}$) high-cholesterol diet (HCD) mice with or without their spleen. Data show GFP$^+$ CD11b cells retrieved from blood and aorta 5 days after transfer. **B**, Enumeration of data above (data shown are pooled from 2 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 GMPs have accumulated in lesions as monocytes and matured to macrophages. **D**, CD45.1 GMPs 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 their merge (yellow) in the intima. **E**, CD45.1 and CD45.2 wild-type (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 (mean±SEM; n=4).

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 with controls, asplenic recipients accumulated fewer GFP$^+$ CD11b$^+$ cells in the blood and aorta (Figure 4A and 4B). In the aorta, among the GFP$^+$ cells, we found differentiated monocytes and 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 HSPCs 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. Strik-

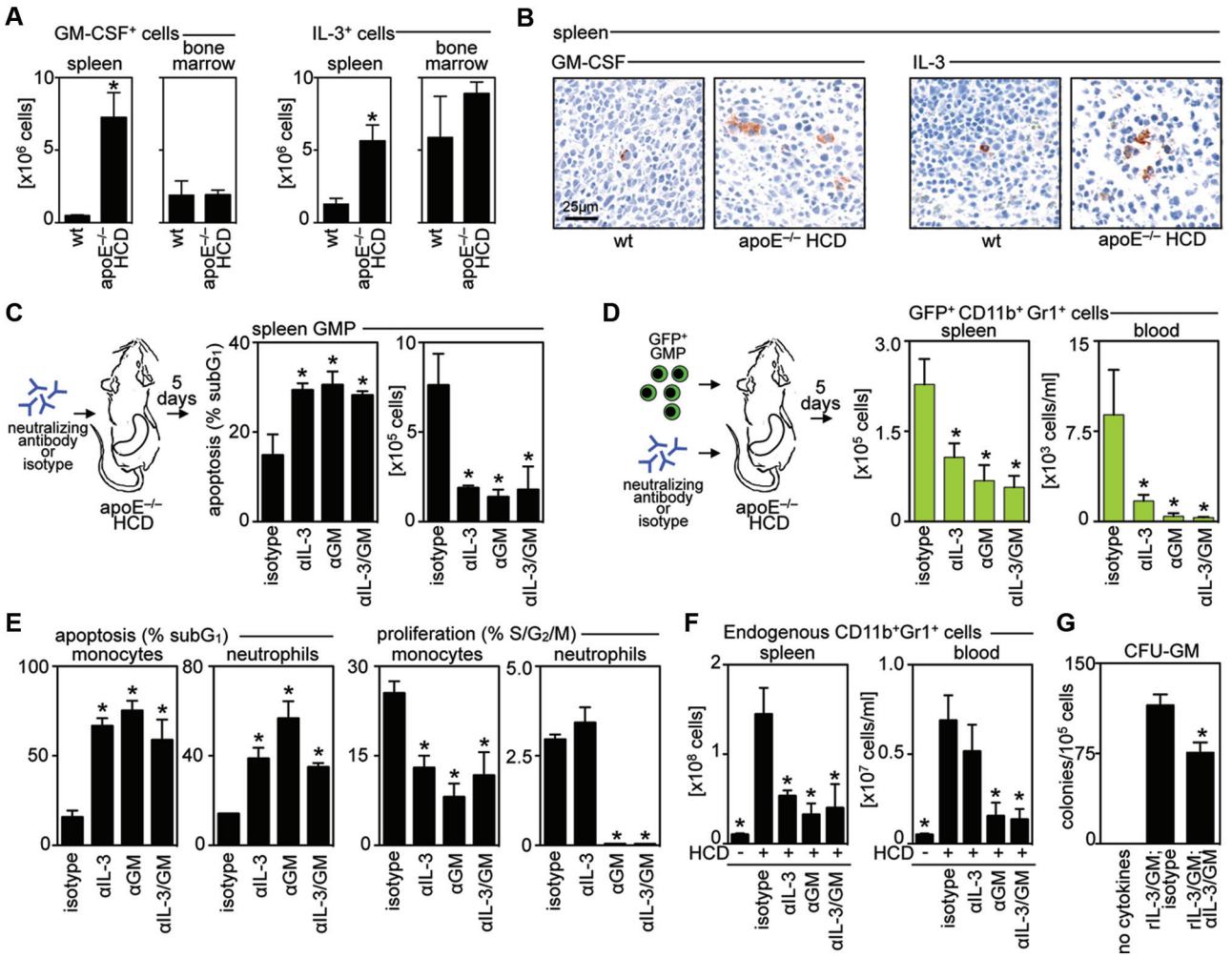

Figure 5. Granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3 control survival and proliferation of myeloid progenitors and progeny in atherosclerosis. **A**, Enumeration of GM-CSF– and IL-3–producing cells by flow cytometry in the spleen and bone marrow in wild-type (wt) and apolipoprotein E–deficient (ApoE$^{-/-}$) high-cholesterol diet (HCD) mice. Data show a preferential increase of GM-CSF and IL-3–producing cells in the spleen (mean±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 granulocyte and macrophage progenitors (GMPs). Data show percent of cells in subG$_1$ and total number of GMPs in spleen (mean±SEM; n=4–5). **D**, Effect of IL-3 and GM-CSF neutralization on the development of myeloid cells after green fluorescent protein—positive (GFP$^+$) GMP pulse chase. Data show GFP$^+$ CD11b$^+$Gr1$^+$ cells in the spleen and blood (mean±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 (mean±SEM; n=4–5). **F**, Effect of IL-3 and GM-CSF neutralization on the endogenous CD11b$^+$Gr1$^+$ repertoire in the spleen and blood (mean±SEM; n=4–5). *$P<0.05$ vs wt (**A**, **B**) or HCD isotype (**C**–**F**). **G**, Colony-forming units granulocyte-macrophage (CFU-GM) 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 (mean±SEM; n=2).

ingly, splenectomy decreased this chimerism by nearly 50%. These data demonstrate, by an independent approach, that the spleen contributes myeloid cells to the atherosclerotic aorta.

Granulocyte Macrophage Colony-Stimulating Factor and IL-3 Promote Survival and Proliferation of Progenitor Cells and Their Progeny

The growth factors granulocyte macrophage colony-stimulating factor (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 Figure IVa in the online-only Data Supplement) and costained for the leukocyte marker CD45 by flow cytometry (Figure IVb in the online-only Data Supplement). 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 (Figure IVc in the online-only Data Supplement). 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 4 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 that, 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 (Figure IVc in the online-only Data Supplement). 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, HSPCs accumulate in the spleen and proliferate in response to GM-CSF and IL-3 and 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 lipopolysaccharide daily for 4 days developed visibly larger spleens (Figure Va in the online-only Data Supplement) containing plenty of monocytes (11-fold expansion over steady state) and neutrophils (23-fold expansion; Figure Vb in the online-only Data Supplement) that resided in the red pulp (Figure Vc in the online-only Data Supplement). To evaluate whether the spleen contributes newly made neutrophils and monocytes in this model, we first enumerated hematopoietic progenitor cells. In response to lipopolysaccharide, GMPs expanded in the spleen (≈62-fold; Figure Vd in the online-only Data Supplement). The progenitors proliferated vigorously (47% were in S or G_2 phase of the cell cycle at a given time; Figure Ve in the online-only Data Supplement) and gave rise to granulocyte macrophage colony-forming units (Figure Vf in the online-only Data Supplement).

We therefore tested whether splenic GMPs give rise to their progeny. Pulse-chase experiments involving the intravenous transfer of GFP- or RFP-expressing GMPs to nonirradiated (wt) C57BL/6 mice that were naïve or that received lipopolysaccharide permitted us to track cell fate by flow cytometry and intravital microscopy. GMPs injected into naïve mice gave rise to only a few progeny (Figure 6A). Injection of GMP to mice that received lipopolysaccharide, 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 5 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 and 6B). Enumeration revealed significant expansion of neutrophils and monocytes in response to lipopolysaccharide 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 lipopolysaccharide. These data show that escalation of the neutrophil and monocyte response in response to endotoxin is associated with an increase in biologically active progenitors in the spleen.

Intravital microscopy showed that GMPs gave rise to clusters of cells that resided outside the 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 (Figure Vg and Vh in the online-only Data Supplement), which is in keeping with the expected lifespan of GMPs.[34] GFP GMPs and RFP GMPs coinjected in equal numbers (Figure 6E) gave rise to clusters that were exclusively red or exclusively green, indicating that GMPs seeded the spleen, proliferated clonally, and differentiated locally (Figure 6F and Figure Vi–Vk in the online-only Data Supplement). 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 tumor necrosis factor-α (Figure 6J). Together, 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 that describes the findings of this study is shown in Figure 7.

Discussion

The motivation for the present study rested on 2 landmark articles published in the 1960s. The first laid the foundation that HSPCs 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: (1) Bone marrow HSPCs mobilize; (2) mobilized HSPCs seed extramedullary sites; and (3) seeded HSPCs proliferate and mature. The cell types that have been described to arise through extramedullary hematopoiesis are terminally differentiated, and many are tissue resident. Mono-

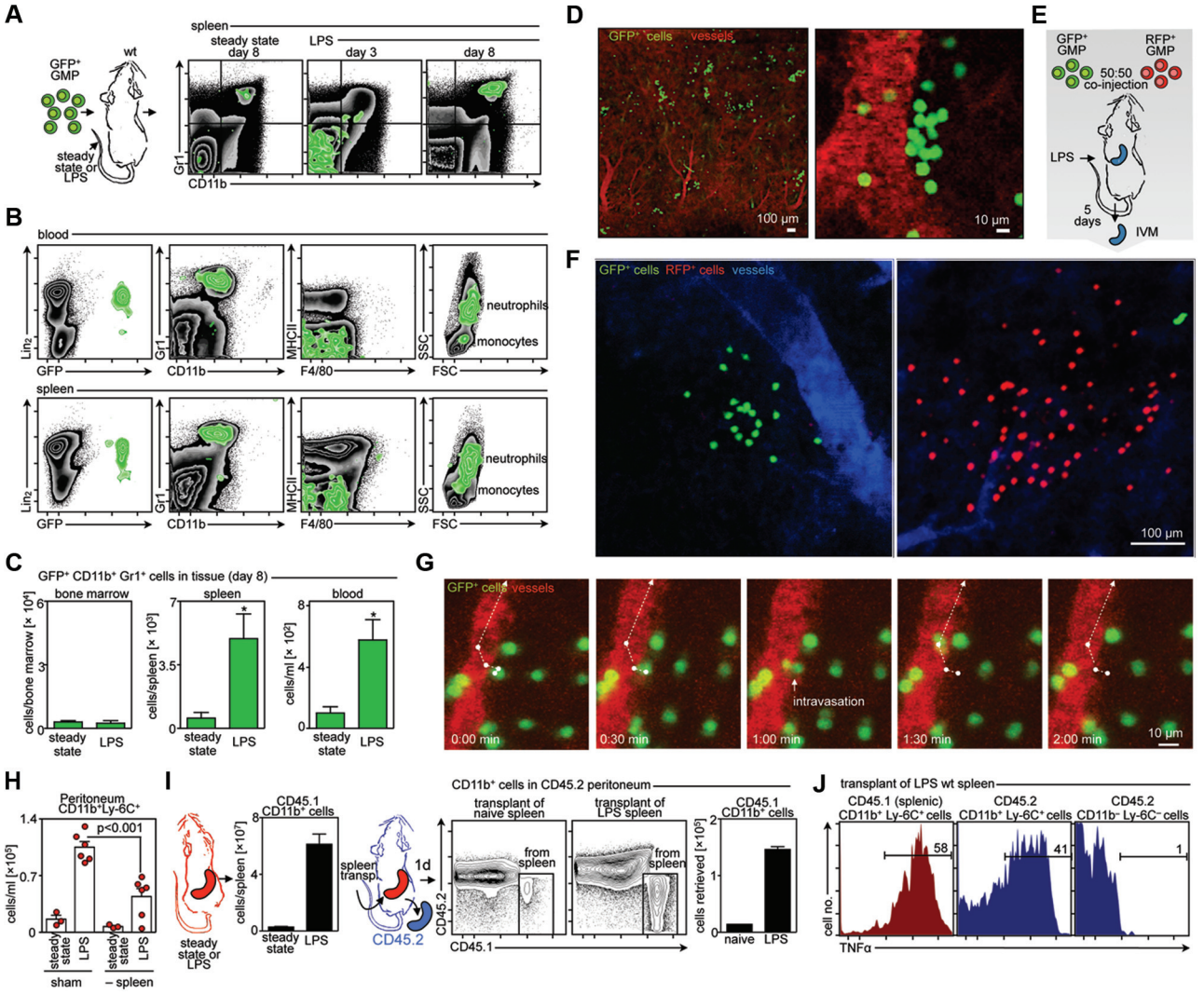

Figure 6. Extramedullary hematopoiesis gives rise to monocytes in response to repeated peritoneal endotoxin challenge. **A**, Cartoon depicts a pulse-chase experiment in which green fluorescent protein—positive (GFP^+) granulocyte and macrophage progenitors (GMPs) were adoptively transferred to wild-type (wt) C57BL/6 mice that remained naïve or were injected with lipopolysaccharide (LPS). A representative plot of at least 3 independent experiments is shown. **B**, GFP^+ GMPs were adoptively transferred to wt C57BL/6 mice injected with LPS. Data are representative of at least 3 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 naïve or inflammatory mice (mean±SEM; n=3–8). $^*P<0.05$. **D**, Intravital microscopy pictograms of the splenic red pulp depict clusters of GFP^+ cells adoptively transferred intravenously 8 days earlier into inflammatory (LPS-injected) mice. Vasculature is shown in red; the scale is depicted with white bars. Data are representative of at least 3 independent experiments. **E**, Cartoon depicts the experimental design for the coinjection of equal numbers of GFP^+ GMPs and red fluorescent protein–positive (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; the scale is depicted by a white bar. Data are representative of at least 3 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 either were naïve or received LPS. The graph shows the total number of splenic $CD11b^+Gr1^+$ that had accumulated in the peritoneum. **J**, Expression of intracellular tumor necrosis factor-α (TNFα). Histograms show TNFα expression on stimulated cells gated on $CD11b^+Gr1^+$ cells of splenic ($CD45.1^+$, red) or other source (ie, bone marrow) ($CD45.2^+$, blue) origin. Data are representative of at least 2 independent experiments.

cytes, on the other hand, are intermediate, circulating cells, developmentally downstream of HSPCs 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 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 fast exchange with the blood,[23] yet it is capable of cell retention through myriad adhesive ligands.[42,43] The organ can accommodate vast quantities and fluctuations of cells, especially in the red pulp. It also allows the rapid exit of undifferentiated monocytes, indicating that entry into its parenchyma neither

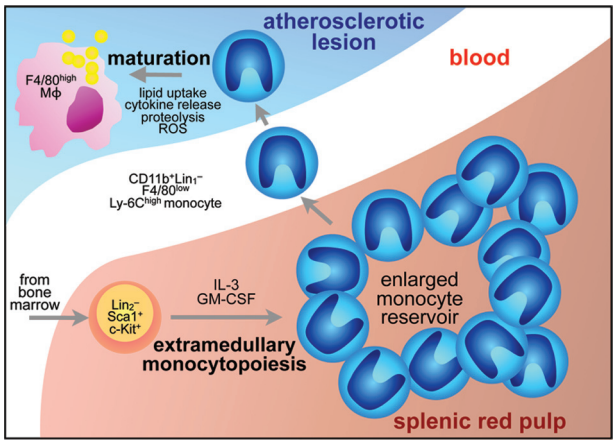

Figure 7. Model depicting the extramedullary generation of monocytes in inflammation. Model shows that mobilized hematopoietic stem and progenitor cells accumulate in the splenic red pulp and give rise to monocytes via interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF). 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. ROS indicates reactive oxygen species.

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 as a result of multiple mechanisms involving platelets, B cells, T cells, and many of the other components that constitute 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 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 occurs during atherosclerosis, when hypercholesterolemia forces a continuous leukocyte supply, is worth further discussion. The steady-state spleen houses a reservoir of undifferentiated monocytes that are, in the absence of neighboring splenic HSPCs, bone marrow derived. The contribution of extramedullary monocytopoiesis during inflammation may be exclusively numeric; 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 and express cytokines, reactive oxygen species, and proteases. They are thus functional and are known to promote the generation of lesions. Future studies 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.

Acknowledgments

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

Sources of Funding

This work was supported in part by National Institutes of Health grants 1R01 HL095612 (Dr Swirski), U01 HL080731, P50 CA86355, U24 CA092782, P01-A154904 (to Dr Weissleder), and 2R01 HL069948 (to Dr Mulligan-Kehoe). Dr Robbins was supported by the American Heart Association postdoctoral fellowship and the Massachusetts General Hospital ECOR Postdoctoral Award. P.J. Rauch was supported by the Boehringer Ingelheim Fonds. Dr Weber was supported by the German Research Foundation.

Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerosis is an inflammatory disease characterized by the accumulation of lipids and leukocytes in the arterial wall. Monocytes are large circulating leukocytes believed to be essential to the development and exacerbation of atherosclerosis. As disease worsens, the number of circulating monocytes rises, whereas in models with monocyte depletion, atherosclerosis neither develops nor evolves. It is believed that hematopoietic progenitors give rise to circulating monocytes exclusively in the bone marrow. These medullary monocytes circulate, accumulate in tissue, and differentiate to macrophages or dendritic cells. Extramedullary sites such as the spleen maintain reservoirs of undifferentiated monocytes that can exit en masse in response to acute inflammation. In this study, we show that during atherosclerosis the bone marrow outsources the production of monocytes to the spleen. These extramedullary monocytes accumulate in the growing atheromata. From a clinical perspective, this finding is important because it identifies the spleen as a possible biomarker organ and therapeutic target for cardiovascular disease, and it proposes that inflammatory hematopoiesis could be targeted therapeutically in atherosclerosis.