Supplemental Material

Expanded Methods and Results

Animals. The LDLR$$^{-/-}$$ApoB48$$^{-/-}$$ mouse was selected because it is reported to more closely mimic human atherosclerosis than other models.\(^1\) ApoE$$^{-/-}$$ animals had been backcrossed to the C57BL/6 background for at least ten generations. ApoE$$^{-/-}$$ CD45.1$$^{+/+}$$ were generated after backcrossing ApoE$$^{-/-}$$ mice to C57BL/6 CD45.1$$^{+/+}$$. At 10 wk of age, ApoE$$^{-/-}$$ mice were placed on a Western diet (21.2% fat/weight; 0.2% cholesterol) (Harlan Teklad, Madison, WI) or remained on a chow diet for durations listed in the manuscript. The LDLR$$^{-/-}$$ApoB48$$^{-/-}$$ mice were placed on Paigen’s high fat diet without cholate (Research Diets, New Brunswick, NJ).\(^2\) for 16 wk at 8 weeks of age. The remaining animals consumed a regular chow diet.

Animal models and in vivo interventions. Splenectomy: Under isofluorane anesthesia, the peritoneal cavity of mice was opened and the splenic vessels were ligated using a 6.0 silk suture. The spleen was then carefully removed. For control experiments, the peritoneum was opened, but the spleen was not excised. The procedure itself does not change the number of circulating monocytes and neutrophils in the steady state\(^3\). Spleen transplantation: Spleen transplantation was conducted as previously described\(^3\). Briefly, spleen donor mice were anesthetized with a subcutaneous injection of ketamine (90 mg/kg) and xylazine (10 mg/kg), followed by an intravenous injection of 200 units of heparin (American Pharmaceutical, Schaumburg, IL). The complete inhibition of clotting ensures that no vascular or intrasplenic thrombosis occurs. In deep anesthesia, the thorax was then opened and the right atrium sectioned to allow blood to exit during perfusion. Over a period of 3 minutes, the entire mouse was then perfused with a total of 15 ml of normal saline through a 25G needle inserted into the apex of the left ventricle. At the end of this procedure, fluid exiting the right atrium was clear which indicates thorough removal of the donor blood. Under isofluorane (1-2%), supplemented with oxygen (1-2 L) anesthesia, the abdomen of the recipient mouse was then opened with a longitudinal incision. The pancreas, the spleen and the abdominal vasculature in the epigastric region were identified and ligated with 6.0 cotton (Ethicon). The celiac artery was then isolated, and the hepatic and gastric artery ligated with 10.0 suture (Ethicon). The abdominal aorta was ligated and cut just below the celiac artery with micro-dissection scissors (ROBOZ, Rockville, MD), and also dissected above the celiac artery. This approach resulted in an aortic cuff
connected to the splenic artery, which allowed vascular anastomosis of the spleen to the
recipient. Following ligation of the bile duct, the portal vein was isolated, and the superior and
inferior mesenteric and gastric veins were ligated. The portal vein was intersected closely to the
liver. The entire organ package containing the vascular connections, spleen and the pancreas
was then removed and stored in ice-cold saline for 15 minutes while the recipient was prepared.
The recipient (CD45.1) was anesthetized with isofluorane (1-2%), supplemented with oxygen
(1-2 L). An abdominal midline incision was made and the inferior vena cava and the descending
aorta isolated below the renal arteries. The recipient vessels were clamped with an atrumatic
vascular clamp (ASSI, Westbury, NY) and opened with micro-scissors. The portal vein was
anastomosed to the inferior vena cava and the donor aortic cuff was connected with an end-to-side anastomosis to the recipient aorta using 10.0 suture. The clamp was then opened to
restore blood flow. Blood flow was confirmed using a blood pool agent (AngioSense-680, VisEn
Medical, MA) and imaging as described previously \(^3\). Parabiosis: The procedure, adapted from
\(^4\) was conducted as previously described\(^3\). Briefly, after shaving the corresponding lateral
aspects of each mouse, matching skin incisions were made from behind the ear to the tail of
each mouse, and the subcutaneous fascia was bluntly dissected to create about \(\frac{1}{2}\) cm of free
skin. The scapulas were sutured using a mono-nylon 5.0 (Ethicon, Albuquerque, NM), and the
dorsal and ventral skins were approximated by continuous suture. After an interval of three
weeks, parabiosed mice were surgically separated by a reversal of the procedure. One group
was splenectomized, as described above. Percent chimerism in the blood was defined for gated
monocytes, neutrophils and Lin\(_1^+\) cells (mostly lymphocytes) as %CD45.1 / (%CD45.1 +
%CD45.2) in CD45.2 mice, and as %CD45.2 / (%CD45.2 + %CD45.1) in CD45.1 mice.
Adoptive transfer of GMP: Donor CD45.1\(^+\) or GFP\(^+\) cells from the bone marrow were first
enriched using anti-PE beads to deplete Lin\(_2^+\) cells (Miltenyi) and then sorted using a BD
FACS\(_\text{Aria II}\) (BD Biosciences). Typically, purity of the sorted population was 99.5 %. \(5 \times 10^4\)
GMPs were injected into the tail vein of non-irradiated recipient mice. Anti-GM-CSF and IL-3
treatment: Mice were i.v. injected with blocking antibody against GM-CSF (MP1-22E9,
eBioscience; 300µg) twice daily for four consecutive days. Control animals received anti-mouse
IgG2a (eBR2a, eBioscience; 300µg). Monocyte reservoir depletion: Mice were i.v. injected with
250 µl clodronate loaded liposomes once. Clodronate was a gift from Roche and was
incorporated into liposomes are described previously \(^5\). Injection of oxLDL: Mice were i.v.
injected once with DiI-oxLDL (Biomedical Technologies, Inc., Stoughton, MA; 100µg).
Endotoxin-induced peritonitis and peritoneal lavage: Mice were administered 10 µg of LPS
(Sigma), unless otherwise stated, daily by i.p. injections in PBS over the course of 4 days. Controls received PBS alone. For select experiments, the peritoneal cavity was lavaged with 10 ml of PBS to retrieve infiltrated and resident leukocytes.

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

were used for flow cytometric analyses in this study. Streptavidin-PerCP was used to label biotinylated antibodies. Cathepsin activity was assessed using Prosense-680 (PerkinElmer). Cell cycle analysis was carried using FxCycle violet stain (Invitrogen). Apoptotic cells were identified as those cells falling within the subG<sub>1</sub> gate as previously described<sup>8</sup>. Contribution of newly-made cells to different cell populations was determined by in-vivo labeling with bromodeoxyuridine (BrdU). Mice received 1 mg of BrdU (BD Biosciences) by i.p. injection. Incorporation was measured using either FITC or APC-conjugated anti-BrdU antibodies according to the manufacturer’s instructions. Monocytes, macrophages/dendritic cells and neutrophils were identified as described previously<sup>3</sup>. Specifically, monocytes were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>-</sup> (Lin<sub>1</sub> = CD90/B220/CD49b/NK1.1/Ly-6G/Ter119) (F4/80/I-A<sup>b</sup>/CD11c)<sup>lo</sup>. Monocyte subsets were identified as either Ly-6C<sup>high</sup> or Ly-6C<sup>low</sup>. Macrophages/dendritic cells were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>-</sup> (F4/80/I-A<sup>b</sup>/CD11c)<sup>hi</sup> or on the basis of F4/80 expression only. Neutrophils were identified as CD11b<sup>hi</sup> Lin<sub>1</sub><sup>-</sup> (F4/80/I-A<sup>b</sup>/CD11c)<sup>lo</sup> Ly-6C<sup>int</sup>. Monocyte and macrophage/dendritic cell numbers were calculated as total cells multiplied by percent cells within the monocyte/macrophage gate. Hematopoietic stem cells were identified as Lin<sub>2</sub><sup>-</sup> (Lin<sub>2</sub> = CD90/B220/CD19/CD49b/Ter119/NK1.1/Gr-1/CD11b/CD11c) IL-7Rα<sup>-</sup> CD117<sup>-</sup> Sca-1<sup>-</sup>. Myeloid progenitors were identified as Lin<sub>2</sub><sup>-</sup> IL-7Rα<sup>-</sup> CD117<sup>+</sup> Sca-1<sup>-</sup>. Within this population, granulocyte/macrophage progenitors (GMP) were CD34<sup>+</sup> CD16/32<sup>+</sup>, common myeloid progenitors (CMP) were CD34<sup>+</sup> CD16/32<sup>+</sup> and megakaryocyte/erythroid progenitors (MEP) were CD34<sup>+</sup> CD16/32<sup>-</sup>. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v8.8.6 (Tree Star, Inc.). Cells were sorted on a BD FACSARia II (BD Biosciences).
**Histology.** Aortae and spleens were excised, embedded in O.C.T. compound (Sakura Finetek), and flash-frozen in isopentane and dry ice. Aortic roots were sectioned into 5 um slices, generating ~30-40 sections that spanned the entirety of the aortic root. For comparison of lesion size between the groups, the sections that captured the maximum lesion area were used. Sections adjacent to this were used for other histological staining. Immunofluorescence staining was carried out using the following primary antibodies: anti-CD11b: clone M1/70 (BD Biosciences), biotin anti-CD45.1: clone A20 (BioLegend), and anti-F4/80: clone CI:A3-1 (Abcam). In some cases, either streptavidin-Texas Red or streptavidin-fluorescein (GE Healthcare) were directly conjugated to primary antibodies. Biotinylated secondary antibodies were used when required. Specificity of staining was confirmed using relevant isotype controls. Cover slips were placed over specimens using mounting medium containing DAPI (Vector Laboratories) to identify cell nuclei. Samples were visualized using an epifluorescence microscope (Nikon Eclipse 80i, Nikon Instruments Inc.) equipped with a Cascade Model 512B camera (Roper Scientific). Immunohistochemical staining was used to detect GM-CSF (clone FL-144, Santa Cruz Biotechnology) and IL-3 (clone MP2-8F8, BioLegend) for spleen sections, and anti-CD11b (clone M1/70, BD Biosciences), anti-Mac3: clone M3/84 (BD Biosciences), anti-F4/80: clone CI:A3-1 (Abcam), and actin smooth muscle (NeoMarkers) for aortic roots. Specificity of staining was confirmed using relevant isotype controls. In order to block endogenous peroxidase activity, tissue sections were pre-treated with 0.3% hydrogen peroxide solution. Following application of appropriate biotinylated secondary antibodies, samples were developed using a Vectastain ABC kit (Vector Laboratories) and AEC substrate (DakoCytomation). All sections were counterstained with Harris Hematoxylin. Masson trichrome (Sigma) and Oil Red O (Sigma) staining were performed to visualize collagen and lipid content, respectively. Hematoxylin and eosin (H&E) staining was performed to assess overall tissue morphology. Captured images were digitized automatically using a Nanozoomer 2.0RS (Hamamatsu).

**Intravital microscopy**

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

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

**(a)** Expression of pro-IL-1β on in vitro-unstimulated Lin⁻CD11b⁺ cells from the aorta of wt and apoE⁻/⁻ mice consuming (HCD) for 20 weeks. Staining for the cytokine is shown as a contour plot and isotype staining is shown as a density plot in the back. Data are representative of at least three independent experiments and show that expression of pro-IL-1β increases in an inflammatory context. **(b)** Isotype staining controls for pro-IL-1β antibody staining in aortic monocytes and macrophages. **(c)** Proteolytic activity on monocytes retrieved from different organs. Data show that monocytes residing in the spleen and bone marrow (bm) have little proteolytic activity. This activity increases when monocytes accumulate in different sites. **(d)**
Uptake of DiI-oxLDL by circulating Lin<sup>-</sup>CD11b<sup>+</sup> cells. Data show that CD45.2<sup>+</sup> (i.e., bone marrow) and CD45.1<sup>+</sup> (i.e. splenic) monocytes take up ox-LDL similarly. DiI-oxLDL was injected i.v. once. (e) Spleen transplantation for 10 days. Data show IF (left panels) on the aortic root with antibodies against CD45.1 (green) and F4/80 (red). DAPI depicts nuclei (blue). Yellow cells represent F480<sup>+</sup> cells of splenic origin. Oil red O staining (right panels) of the same aortic root tissue section reveals co-localization of lipid accumulation and lesional cells of splenic origin. (f) H&E staining of aortic root lesions of spleen-containing and asplenic apoE<sup>−/−</sup> HCD mice. (g) Immunohistochemical staining for macrophage/monocyte markers in aortic root sections from mice in (f). Depicted is representative staining for Mac3, CD11b, and F4/80. (h) Masson trichrome staining of aortic roots from representative control and splenectomized mice. (i) Actin smooth muscle staining.

Supplemental Figure III
(a) Enumeration of Lin<sub>2</sub>Sca<sub>1</sub>−c-kit<sup>+</sup> (LSK), common myeloid progenitor (CLP), granulocyte-macrophage precursors (GMP), and common lymphoid progenitor cells (CLP) in spleens of C57BL/6 (wt) and apoE<sup>−/−</sup> mice consuming a high cholesterol diet (HCD) for 8 weeks. Data show that myeloid progenitor populations, but not lineage progenitor cells increase in number in apoE<sup>−/−</sup> mice consuming a HCD. (b) Enumeration of GMP in the spleens of LDLR<sup>−/−</sup>ApoB48<sup>−/−</sup> mice consuming Paigen's diet. (means ± SEM, n = 5-8) *P< 0.05. (c) Representative post-sort
flow cytometric analysis of GMPs that were used for adoptive transfer. Overall purity of GMPs within the living population was 99.5%. This is a quality control experiment showing that the GFP+ GMP that are injected are sorted to high purity. (d) Adoptive transfer of GFP+ GMP to wt and apoE−/− HCD mice. Data show GFP cells in spleens 3 days after transfer. Increased percentage of GFP+ progenitors and myeloid cells was observed in spleens of apoE−/− HCD mice.

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

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

References (to Expanded Methods)