Atherosclerosis is regarded as a chronic inflammatory disease of the vessel wall, characterized by an intimal accumulation of immune cells at sites of plaque formation, leading to the progressive narrowing of the arterial lumen. A prominent role herein has been ascribed to macrophages, which accumulate oxidized low-density lipoprotein (oxLDL) and other lipids and transform into foam cells within the arterial wall. However, polymorphonuclear neutrophilic leukocytes (PMN) constituting the most abundant white blood cells in the circulation have also recently been associated with the development of atherosclerosis. Although systemic PMN counts correlate with the severity of atherosclerosis and PMN localize at sites of plaque rupture and erosion in patients with acute coronary syndromes and symptomatic plaques, PMN were also localized in murine atherosclerotic plaques, and increased peripheral PMN numbers associated with aggravated atherosclerosis and inflammation in murine models of atherosclerosis.

Interferon regulatory factor 8 (IRF8) (also known as interferon consensus sequence binding protein) is exclusively expressed in hematopoietic cells and critical in lineage determination and the development of myeloid cells from common progenitor cells. Although essential in directing monocyte and macrophage differentiation, IRF8 represses genes promoting granulocytic differentiation and maturation. In consequence, IRF8-deficient (IRF8−/−) or IRF8 mutant mice develop a chronic myelogenous leukemia (CML)-like syndrome with a striking increase in PMN numbers whereas cells bearing monocyte/macrophage markers are reduced and functionally compromised.
Given the important contribution of PMN to atherosclerosis and neutrophilia to be a hallmark of human CML, we addressed atherosclerotic lesion formation in atherosclerosis-prone IRF8−/− mice in hematopoietic cells that develop a CML-like disease.

Methods
Please see Methods in the online-only Data Supplement.

Mouse Models
Six- to 8-week-old female recipient apolipoprotein E-deficient (apoE−/−) or LDL receptor-deficient (LDLR−/−) mice were transplanted with bone marrow (BM) cells from IRF8−/−, IRF8+−/−, or IRF8−/− apoE−/− mice (all C57BL/6 background) after an ablative dose of whole body irradiation, and were fed a standard western diet containing 21% fat and 0.15% cholesterol (Altromin). PMN were depleted by intraperitoneal injections of monoclonal antibody RB6-8C5 every other day. A subcutaneous air pouch was created in IRF8−/− or IRF8+−/− mice, and injected with platelet-activating factor. Peritonitis was induced by intraperitoneal injection with thioglycolate. Cells were collected from lavage fluids, and processed for fluorescence activated cell sorter (FACS) analysis.

Atherosclerotic Lesion Quantification and Immunohistochemistry
Atherosclerosis and lipid accumulation were assessed in aortic roots and thoracoabdominal aortas by staining for lipid depositions with Oil Red O (Figure I in the online-only Data Supplement). The relative content of macrophages, T cells, smooth muscle cells, and PMN were determined by staining with specific monoclonal antibody or appropriate isotype controls. Apoptotic nuclei were detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Collagen was stained using Sirius Red.

Flow Cytometry, Cell Sorting, and ELISA
After staining with directly conjugated antibodies, FACS analysis and cell sorting were performed using a FACSCanto II or FACSARia. Interleukin-10 (IL-10) concentrations were determined using the mouse IL-10 DuoSet ELISA Kit (R&D).

Analysis of Myeloperoxidase and Matrix Metalloproteinase-9 Activity and Reactive Oxygen Species Formation
Myeloperoxidase (MPO) activity was measured as described. Matrix metalloproteinase (MMP)-9 activity was quantified using the Sensolyte MMP-9 assay kit and reactive oxygen species (ROS) formation determined by analyzing the fluorescence of PMN loaded with 2′,7′-dichlorodihydrofluorescein diacetate before and after tumor necrosis factor-α or phorbol myristate acetate stimulation.

Apoptosis, Cell Cycle Assays, and Phagocytosis
Cell staining with Annexin V or propidium iodide and the uptake of 1,1′-dioctadecyl-3,3′,3′-tetra-methylindocyanide perchlorate–labeled oxLDL or calcine-labeled apoptotic PMN by peritoneal macrophages were analyzed by FACS.

Statistics
Data are represented as mean±SD, and were analyzed by Student t test, ANOVA with Tukey multiple comparison test, nonparametric Mann-Whitney test, or Kruskal-Wallis test with Dunn post hoc test (Prism 4.0 software, GraphPad), as appropriate. Data for ROS measurement were analyzed with 2-way repeated measures ANOVA (treatment time) followed by planned comparisons. P<0.05 was considered to be statistically significant.

Results
ApoE−/− Mice Transplanted With IRF8−/− BM Display a CML-Like Phenotype
Due to the exclusive expression of IRF8 in hematopoietic cells, we chose to transplant IRF8−/− BM into atherosclerosis-prone apoE−/− mice. Similar to the distribution of peripheral blood cells in donor IRF8−/− mice (not shown), lethally irradiated apoE−/− mice reconstituted with IRF8−/− BM displayed a trend for expansion in the number of leukocytes and a significant increase in the number and frequency of PMN in peripheral blood, whereas absolute counts of circulating total monocytes, encompassing both the Ly6C(high) and Ly6C(low) (Gr-1low) monocyte subset, were reduced (Figure 1A–1C). Circulating numbers of dendritic cells, T cells and B cells were unaltered in apoE−/− mice reconstituted with IRF8−/− BM (data not shown). Because IRF8−/− monocytes show only marginal expression of CD115, possibly related to its enhanced proteolytic degradation, monocytes were identified by staining for CD11b and Gr-1high but the absence of PMN-rich blasts. Similar shifts in the distribution of leukocyte subsets were observed in the BM of transplanted mice, with an even more pronounced expansion of PMN relative to macrophages, smooth muscle cells, and thoracoabdominal aortas by staining for lipid depositions with Oil Red O (Figure I in the online-only Data Supplement). Quantifications of lipid depositions within lesions revealed a shift toward enhanced accumulation of extracellular in contrast to intracellular lipids in apoE−/− mice carrying IRF8−/− compared with IRF8+−/− BM (Figure IIA and IIB in the online-only Data Supplement). Because apoE−/− mice carrying IRF8−/− or IRF8+−/− BM (not shown, Figure 1D). This is in line with a chronic disease course with no signs of an accumulation of progenitor or immature myeloid cells in blood, characteristic of the late progressive blast phase.

Increased Atherosclerotic Lesion Formation in ApoE−/− Mice Transplanted With IRF8−/− BM
Atherosclerotic lesion formation was analyzed after 12 weeks of high-fat diet feeding. The quantification of lipid depositions in the aorta and aortic roots revealed a significant exacerbation in lesion formation in apoE−/− mice reconstituted with IRF8−/− compared with IRF8+−/− BM (Figure 2A and 2B). In additional control experiments, we similarly found an increased atherosclerotic lesion formation in apoE−/− mice reconstituted with IRF8−/−apoE−/− in comparison with IRF8+−/−apoE−/− BM (Figure 2C). Increased atherosclerotic lesion formation was confirmed by the increased lesion formation in apoE−/− mice carrying IRF8−/− or IRF8+−/− BM (not shown, Figure 1D).

Increased atherosclerotic lesion formation was analyzed after 12 weeks of high-fat diet feeding. The quantification of lipid depositions in the aorta and aortic roots revealed a significant exacerbation in lesion formation in apoE−/− mice reconstituted with IRF8−/− compared with IRF8+−/− BM (Figure 2A and 2B). In additional control experiments, we similarly found an increased atherosclerotic lesion formation in apoE−/− mice reconstituted with IRF8−/−apoE−/− in comparison with IRF8+−/−apoE−/− BM (Figure 2C). Increased atherosclerotic lesion formation was confirmed by the increased lesion formation in apoE−/− mice carrying IRF8−/− or IRF8+−/− BM (not shown, Figure 1D). This is in line with a chronic disease course with no signs of an accumulation of progenitor or immature myeloid cells in blood, characteristic of the late progressive blast phase.

Total serum cholesterol, very low-density lipoprotein/ LDL, and high-density lipoprotein levels were unaltered in apoE−/− mice reconstituted with IRF8−/− versus IRF8+−/− BM, and IRF8−/−apoE−/− versus IRF8+−/−apoE−/− BM (Table I in the online-only Data Supplement). Quantifications of lipid depositions within lesions revealed a shift toward enhanced accumulation of extracellular in contrast to intracellular lipids in apoE−/− mice carrying IRF8−/− compared with IRF8+−/− BM (49.8±1.5 versus 31.6±3.6% extracellular Oil Red O+ lipid deposits, respectively, P<0.01). Analysis of the cellular plaque composition by quantitative immunofluorescence further showed that the number of macrophages, smooth muscle cells,
and T cells was not altered in aortic root plaques whereas a significant increase in the relative number of Ly6G+ PMN was observed in lesions of apoE<sup>−/−</sup> mice with IRF8<sup>−/−</sup> BM (Figure 2C). Moreover, the expression of MPO, an enzyme stored in PMN granules, as well as the frequency of MMP-9+ cells<sup>10,11,23</sup> was found to be increased (Figure IIIA and IIIB in the online-only Data Supplement), whereas the content of collagen-rich extracellular matrix, evident by Sirius Red staining, was reduced in lesions of apoE<sup>−/−</sup> mice reconstituted with IRF8<sup>−/−</sup> compared with IRF8<sup>+/+</sup> BM (Figure IIIC in the online-only Data Supplement), implying a more inflammatory and unstable plaque phenotype. In agreement with this notion, the expression of intracellular adhesion molecule 1 was increased in plaques of apoE<sup>−/−</sup> mice reconstituted with IRF8<sup>−/−</sup> BM (Figure IV in the online-only Data Supplement). Notably, the sizes of necrotic cores in atherosclerotic lesions were significantly larger in apoE<sup>−/−</sup> mice reconstituted with IRF8<sup>−/−</sup> and IRF8<sup>−/−</sup> apoE<sup>−/−</sup> BM in comparison with respective controls (Figure 2D; Figure IIC in the online-only Data Supplement), and number of lesional TUNEL<sup>+</sup> apoptotic cells was increased in apoE<sup>−/−</sup> mice carrying IRF8<sup>−/−</sup> BM (Figure 2D). To further unravel the cell type undergoing apoptosis, double immunofluorescence staining was performed. Although the macrophage fraction within TUNEL<sup>+</sup> cells did not differ, a clear increase in relative numbers of TUNEL<sup>+</sup> Ly6G<sup>+</sup> PMN could be detected in apoE<sup>−/−</sup> mice with IRF8<sup>−/−</sup> versus IRF8<sup>+/+</sup> BM (Figure 2E and 2F). These results suggest that exacerbated atherosclerotic lesion formation in IRF8<sup>−/−</sup> BM-recipient apoE<sup>−/−</sup> mice is associated with an enhanced accumulation and subsequent apoptosis of PMN within growing lesions.

**Impairment of Macrophage Function But Not Accumulation in IRF8<sup>−/−</sup> Mice**

Despite an increase in the total number of leukocytes accumulating in air pouches of IRF8<sup>−/−</sup> mice in response...
Figure 2. Increased atherosclerotic lesion formation and necrotic cores in interferon regulatory factor 8-deficient (IRF8−/−) bone marrow (BM)-recipient apolipoprotein E-deficient (apoE−/−) mice. A and B, Quantification of Oil Red O+ lipid depositions in the aorta (A) and aortic root (B) of apoE−/− mice reconstituted with IRF8−/− (n=10) and IRF8+/+ BM (n=7); representative images. C, Quantification of macrophage (n=5–6), and smooth muscle cell (SMC) content (n=5–7), T cells (n=4–7), and polymorphonuclear neutrophilic leukocytes (PMN; n=6 each) in aortic root plaques. D, Quantification of acellular necrotic cores (n=7–9 each) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL+) apoptotic cells (n=7–8 each) within aortic root lesions of apoE−/− mice reconstituted with IRF8−/− and IRF8+/+ BM. E, Quantification of double-positive TUNEL+ macrophages and TUNEL+ PMN relative to total TUNEL+ cells (n=7 each). F, Representative images of Ly6G and TUNEL double immunofluorescence staining. Cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI). *P<0.05, **P<0.01, ***P<0.001. MOMA-2 indicates monocyte/macrophage marker.
to platelet-activating factor after 4 hours and a significant decrease in relative frequencies of monocytes, the absolute number of monocytes extravasated into the air pouch did not differ in IRF8\(^{+/+}\) or IRF8\(^{-/-}\) mice, contrasting decreased monocyte counts in peripheral blood (Figure 3A and 3B). Likewise, no differences in macrophage numbers were observed in the peritoneum of untreated mice or at 4 days after thioglycollate injection when comparing IRF8\(^{+/+}\) and IRF8\(^{-/-}\) mice (Figure 3C). The rate of apoptosis and proliferation of peritoneal macrophages did not differ between IRF8\(^{-/-}\) and IRF8\(^{+/+}\) mice, and was not altered in response to oxLDL, a stimulus relevant in atherogenesis (Figure V in the online-only Data Supplement). These data suggest that inflammatory monocyte recruitment in the acute or chronic setting and apoptosis of macrophages is not impaired by IRF8 deficiency.

To address an IRF8-related impact on macrophage phenotype, we further analyzed the surface expression of major histocompatibility complex class II, costimulatory molecule CD40 and scavenger receptors in peritoneal macrophages from IRF8\(^{+/+}\) or IRF8\(^{-/-}\) mice by FACS analysis. The expression of major histocompatibility complex class II, CD40, and surface class B scavenger receptor CD36 was significantly decreased in IRF8\(^{-/-}\) macrophages compared with IRF8\(^{+/+}\) controls whereas the expression of class A scavenger receptors CD68 and CD204 was not affected (Figure 4A and 4B). In accordance with a prominent role of CD36 in the uptake of oxLDL and the clearance of apoptotic cells,\(^{24,25}\) a significantly decreased phagocytosis of labeled oxLDL and efferocytosis of apoptotic PMN was observed in IRF8\(^{-/-}\) in comparison with IRF8\(^{+/+}\) macrophages, as determined by FACS analysis (Figure 4C). Phagocytosis of apoptotic cells was shown to exert immunosuppressive effects by inducing the production of anti-inflammatory cytokines including IL-10 in phagocytes.\(^{26,27}\) Compared with a strong secretion of IL-10 by IRF8\(^{+/+}\) macrophages in response to the uptake of apoptotic PMN, the release of IL-10 was substantially impaired in IRF8\(^{-/-}\) macrophages in line with diminished phagocytic capacities (Figure 4D). These differences may also relate to reduced IL-10 serum levels in apoE\(^{-/-}\) mice transplanted with IRF8\(^{+/+}\) BM compared with controls (Figure VIA in the online-only Data Supplement). Given the low frequencies of classic dendritic cells and T cells (Figure VIB in the online-only Data Supplement) and a lack of regulatory T cells in air pouch lavage fluids (not shown), we conclude that these data point toward a defect in IL-10 production by IRF8\(^{-/-}\) macrophages.

**Inflammatory Accumulation of IRF8\(^{-/-}\) PMN Is Due to Enhanced Extravasation**

The enhanced frequency of PMN in apoE\(^{-/-}\) mice reconstituted with IRF8\(^{-/-}\) BM in aortic root plaques led us to further address the recruitment of PMN. Similarly, the number of emigrated PMN in the air pouch lavage in response to platelet-activating factor was significantly increased in IRF8\(^{-/-}\) mice after 4 hours (Figure 5A). However, the ratio of PMN numbers in blood relative to air pouches was identical in either strain (0.37±0.04 in IRF8\(^{-/-}\) versus 0.40±0.09 in IRF8\(^{+/+}\) mice), suggesting that PMN are recruited with similar efficiencies and that their enhanced accumulation at sites of inflammation in mice with IRF8\(^{-/-}\) BM reflects an influx proportional to their numbers in blood.

An increased frequency of apoptotic PMN in apoE\(^{-/-}\) mice reconstituted with IRF8\(^{-/-}\) BM (Figure 2) may indicate an enhanced susceptibility of IRF8\(^{-/-}\) PMN to undergo cell death. Although both blood and air pouch-derived IRF8\(^{-/-}\) PMN tended to undergo apoptosis less frequently early after the induction of serum starvation, no differences could be detected after 36 hours with \(\geq 50\%\)-Annexin V\(^{+}\) apoptotic PMN, and at later time points, as revealed by FACS analysis and when compared with IRF8\(^{+/+}\) PMN (Figure 5B). Of note, BM-derived IRF8\(^{-/-}\) PMN were less susceptible to apoptosis compared with IRF8\(^{+/+}\) controls (not shown), confirming previous findings.\(^{16}\) Enhanced proliferation of immature extravasated PMN may
yet be another mechanism contributing to PMN accumulation in myeloproliferation. Cell cycle analysis of PMN from blood and air pouches of IRF8+/+ and IRF8−/− mice, however, did not reveal any differences in the number of proliferating cells in the synthesis and mitosis phase, excluding this possibility (Figure 5C). This indicates that apoptosis and proliferation of PMN in blood and at sites of inflammation are not substantially affected by the lack of IRF8. Thus, increased numbers of PMN and TUNEL+ PMN in atherosclerotic lesions are likely due to an overflow of extravasated PMN.

**ROS Formation and Granule Discharge Are Not Impaired in IRF8−/− PMN**

ROS play a central role in the development of atherosclerosis, and initiate and sustain key mechanisms of atheroprogression.28 Because PMN are a major source of ROS, we assessed the ROS-forming aptitude of peripheral PMN sorted from blood and BM. Both the treatment with tumor necrosis factor or phorbol myristate acetate triggered an equal release of ROS over baseline in IRF8+/+ compared with IRF8−/− PMN (Figure 5D).

PMN contain preformed granules, which are rapidly released on extravasation. This process can be assessed by the upregulation of membrane-bound receptors translocated from the granule membrane or by measurement of granule proteins in the cell-free supernatant.18,20,29 Compared with circulating blood PMN, the surface expression of CD11b and CD14 derived from secretory vesicles and tertiary granules29 were markedly upregulated on both IRF8+/+ and IRF8−/− PMN after extravasation to the air pouch (Figure 5E). Concentrations of MMP-9 and MPO, which are stored in primary and secondary PMN granules,29 were found to be significantly increased in the air pouch lavage from IRF8−/− compared with IRF8+/+ mice (Figure 5F, left). However, no significant differences in the amount of enzymes released per PMN were observed between both strains in the air pouch (Figure 5F, right). This indicates that IRF8−/− PMN are functionally intact, and that enhanced numbers of extravasated PMN likely contribute to an overall increased ROS- and granule-releasing capacity.

**PMN Depletion in IRF8−/− BM Recipients Prevents Aggravated Atherosclerotic Lesion Formation**

To scrutinize the contribution of PMN to aggravated lesion formation in mice reconstituted with IRF8−/− BM, a PMN-depleting antibody was used (Figure VII in the online-only Data Supplement). Because neutropenia by repeated antibody injections can only be sustained for 4 to 5 weeks, we chose a shorter observation period for these experiments. Recapitulating results obtained in transplanted apoE−/− mice, LDLR−/− mice reconstituted with IRF8−/− BM displayed an expansion of leukocytes and neutrophils (not shown) and significantly increased early atherosclerotic lesion formation in aortas and aortic roots after 5 weeks of high-fat diet compared with control LDLR−/− mice carrying IRF8+/+ BM (Figure 6A and 6B). Importantly, depletion of PMN prevented the exacerbation of PMN accumulation and elevated atherosclerotic lesion formation in IRF8−/− BM recipients. This indicates that PMN contribute to the development of atherosclerotic lesions in the absence of IRF8.
Figure 5. Accumulation of functionally intact polymorphonuclear neutrophilic leukocytes (PMN) in interferon regulatory factor 8-deficient (IRF8−/−) mice is due to their enhanced extravasation. A, Representative fluorescence activated cell sorter (FACS) dot plots of PMN (gated events, inserted numbers indicate percentages relative to CD45+ cells) in blood and air pouch lavages 4 hours after platelet-activating factor (PAF) stimulation in IRF8+/+ and IRF8−/− mice. Absolute PMN counts in blood and air pouch lavages are depicted in bar graphs (n=5–6 each). B, FACS analysis of the frequencies of Annexin V+ apoptotic and (C) propidium iodide (PI)+ proliferation cells in the synthesis and mitosis phase as determined by cell cycle analysis after intracellular staining in PMN sorted from blood and air pouch lavages of IRF8+/+ and IRF8−/− mice and after serum starvation for indicated time (n=5 each). D, Reactive oxygen species (ROS) release from PMN sorted from peripheral blood of IRF8+/+ and IRF8−/− mice in response to tumor necrosis factor (TNF) or phorbol myristate acetate (PMA) in comparison with untreated cells. Lines represent mean values (n=5 each). E, FACS analysis of the surface expression of CD11b and CD14 in PMN in blood (red line) and air pouches (black line) of IRF8+/+ and IRF8−/− mice. Filled histograms (gray) represent fluorescence minus one (FMO) controls. One representative histogram of 5 independent experiments is shown. F, Activity of myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) in air pouch lavages collected from IRF8+/+ (n=5) and IRF8−/− mice (n=6) after 4 hours of PAF stimulation and PMN extravasation. Values are expressed on a per volume (left) and on a per cell basis (right). *P<0.05, **P<0.01, ***P<0.001. MFI indicates mean fluorescence intensity.
in lesion formation in LDLR−/− mice reconstituted with IRF8−/− BM, and in apoE−/− mice transplanted with IRF8−/−apoE−/− BM (Figure 6A and 6B; Figure VIIIA and VIIIB in the online-only Data Supplement). Although macrophage numbers in atherosclerotic lesions were not affected in either group, the enhanced accumulation of Ly6G+ PMN and TUNEL+ cells in IRF8−/− BM-recipient LDLR−/− mice was prevented by treatment with the PMN-depleting antibody (Figure 6C; Figure VIIIC in the online-only Data Supplement; data not shown). Furthermore, the increase in necrotic core formation in LDLR−/− mice with IRF8−/− BM and in apoE−/− mice with IRF8−/−apoE−/− BM was prevented when mice were rendered neutropenic, with necrotic core sizes remaining similar to respective IRF8−/− BM controls (Figure 6C; Figure VIIIC in the online-only Data Supplement). No changes in serum lipid levels were observed between the groups (Table I in the online-only Data Supplement). These data establish that the expansion of PMN in blood and at sites of inflammation critically contributes to atherosclerotic lesion formation in mice with IRF8−/− BM.

Discussion

In this article, we show that the development of a CML-like syndrome in apoE−/− and LDLR−/− mice transplanted with IRF8−/− or IRF8−/−apoE−/− BM is associated with a striking increase in atherosclerotic lesion formation compared with mice reconstituted with IRF8−/+ control BM. The marked expansion of functionally intact PMN in peripheral blood was accompanied by their enhanced accumulation but also apoptosis at sites of inflammation and an accelerated necrotic core formation and plaque growth in IRF8−/− BM recipients. IRF8−/− PMN were functionally intact, entailing an enhanced release of ROS and granule components at sites of inflammation. Despite a reduction in circulating monocyte numbers, the accumulation of macrophages during inflammation was unaffected by IRF8 deficiency, but associated with an altered macrophage phenotype, as revealed by a diminished efferocytosis of apoptotic PMN and an impaired IL-10 cytokine release. By demonstrating that the depletion of circulating PMN retarded necrotic core formation and prevented the exacerbation of plaque growth in IRF8−/− BM-recipient LDLR−/− or apoE−/− mice, we pinpointed PMN to critically contribute to accelerated atherosclerotic lesion formation in CML-like disease. Although lesion formation was attenuated in apoE−/− mice transplanted with apoE−/− compared with apoE−/− BM, supporting the notion that the secretion of apoE by BM-derived macrophages protects apoE−/− mice from diet-induced atherosclerosis, deficiency in IRF8 in BM consistently resulted in accelerated lesion formation irrespective of the mouse model used, strengthening the general validity of our findings. Emerging evidence suggests that PMN play a critical role in atherosclerosis.1–12,30,31 We have previously shown that increased numbers of circulating and lesional neutrophils aggravated diet-induced atherosclerosis in apoE−/− and LDLR−/− mice.10 Moreover, neutrophilia induced by hyperlipidemia could be related to accelerated atherosclerotic lesion formation in apoE−/− mice.12 In these studies, depletion of neutrophils reversed early atherosclerotic lesion formation, establishing an important role of this cell type in atherosclerosis. Similarly, transplantable myeloproliferative disease in ATP-binding cassette transporter-deficient Abca1−/− and Abcg1−/− mice, associated with leukocytosis and neutrophilia, enhanced atherosclerosis.13 The accumulation of neutrophils in human atherosclerotic plaques associates with characteristics of rupture-prone lesions,29 and circulating PMN counts correlate with cardiovascular complications.30,31

It remains to be elucidated, however, whether patients with myeloproliferative diseases such as CML and long-standing neutrophilia would be more prone to atherosclerosis. Clinical reports on the association of CML with atherosclerosis or its consequences are scarce. One study including a small number of patients found an association of coronary artery disease with coexisting myeloproliferative syndromes, which was not significant after adjustment for various risk factors.32 Several reasons may account for the limited insight into potential consequences of CML-like disease on atherosclerosis in patients. CML is an age-related disease with an increased prevalence in elderly people. These patients may already suffer from cardiovascular complications of multifactorial origins, making it impossible to investigate the impact of CML as an independent risk factor. In addition, effective treatment regimens for CML are available. Decreased IRF8 expression was demonstrated in both humans and mice with CML.36,37 Interestingly, a mutation of the IRF8 gene was recently associated with high neutrophil counts and an absence of monocytes,38 resembling the phenotype of mice with loss of IRF8. Therapeutics often aim at elevating IRF8 expression, eg, treatment with interferon-α stimulates the expression of IRF8, and in consequence partially reverses CML, and therapy with imatinib mesylate inhibits the tyrosine kinase activity encoded by the breakpoint cluster region-Abelson (BCR-ABL) fusion gene and subsequently restores IRF8 expression, inducing a complete cytogenic response in 80% of patients with CML in the chronic phase.39 In this regard, it is important to note that treatment with imatinib was shown to reduce diabetes mellitus–associated atherosclerosis in mice,40 and preliminary experiments indicate that administration of imatinib lowered the numbers of PMN in peripheral blood of BL6 mice (Yvonne Döring and Alma Zernecke, unpublished observation, 2010).

The immune cell infiltration of the vessel wall is a crucial force driving atherosclerosis.1–4 Through the expression of scavenger receptors, macrophages participate in the phagocytic uptake of lipids and their retention in the vessel wall, as well as in the clearance of apoptotic cells.2,3,24,25 Although surface expression of scavenger receptors CD68 and CD204 was unaltered, CD36 was markedly reduced in IRF8−/− macrophages. Known to account for up to 70% of the accumulation of oxLDL in macrophage foam cells,24 these differences might at least, in part, explain the impaired uptake of oxLDL by IRF8−/− macrophages in vitro, and an increased accumulation of extracellular lipid deposits in plaques of IRF8−/− BM-recipient mice. Interestingly, although deletion of CD36 was shown to protect from atherosclerosis,40 an impaired clearance of apoptotic material has been associated with disease acceleration and a reduction in IL-10 production.36 Accordingly, diminished IL-10 protein levels were observed in the supernatants of IRF8−/− macrophages in response to an impaired efferocytosis of apoptotic PMN and in sera of apoE−/− mice carrying IRF8−/− BM.
The depletion of monocytes/macrophages in mice expressing the diphtheria toxin receptor under the control of CD11b or the reduction in blood monocyte levels due to the lack of important mobilization or survival cues was shown to reduce early plaque burden. However, despite a marked reduction in circulating monocyte numbers in mice with IRF8−/− BM, unaltered acute and chronic recruitment of monocytes to the inflammatory air pouch and peritoneum, and unchanged macrophage plaque content were observed. This likely precludes severe functional deficits in the migratory capacity of IRF8−/− monocytes per se. An enhanced survival of IRF8−/− macrophages masking defects in extravasation is unlikely to occur under inflammatory conditions, because previous findings showed a reduction in the susceptibility to apoptosis in IRF8−/− myeloid cells under homeostatic conditions but not on inflammatory stimulation. Accordingly, we did not detect any differences in the frequency of apoptosis in IRF8−/− macrophages in the peritoneum and in response to oxLDL in vitro, and in atherosclerotic lesions of apoE−/− mice reconstituted with IRF8−/− BM.

However, augmented PMN extravasation and PMN-induced inflammation in mice reconstituted with IRF8−/− BM may provide excessive recruitment cues that enhance subsequent monocyte influx. For instance, ROS formation was unaltered in IRF8−/− PMN, constituting a weapon being at the immediate demand of PMN and proposed to be a key mediator in atherogenesis. PMN produce large amounts of ROS via MPO, lipoxygenases, and nicotinamide adenine dinucleotide phosphate oxidase, in addition to triggering the production of pro-inflammatory cytokines, PMN-derived ROS may act through an enhancement of the expression of endothelial adhesion molecules (contributing to leukocyte recruitment), the induction of apoptosis of endothelial cells, or lipid oxidation within lesions (with oxLDL functioning as a strong proinflammatory mediator). This enhanced inflammatory environment within plaques of IRF8−/− BM-recipient mice, as also epitomized by an increased expression of the intercellular adhesion molecule 1, may drive monocyte recruitment to lesions and compensate for reduced peripheral monocyte counts.

In addition, emigrated PMN release preformed granule components, which can trigger the subsequent recruitment and attraction of inflammatory monocytes by involvement of formyl peptide receptors but can also promote proinflammatory responses in monocytes/macrophages. Monocytes/macrophages accumulating at sites of inflammation may thus contribute to accelerated atherogenesis in mice reconstituted with IRF8−/− BM.

The activation of PMN and the discharge of granule proteins were not impaired, as determined by the rapid upregulation of CD11b and CD14 on extravasated IRF8−/− BM, or by the release of MPO and MMP-9 from primary and secondary granules. MPO can limit the bioavailability of NO, and can therefore contribute to the onset of endothelial dysfunction. It was also reported that MPO-generated hypochlorous acid may contribute to intracoronary endothelial cell desquamation and the engendement of a prothrombotic phenotype. Due to its proteolytic function, MMP-9 was furthermore shown to contribute to the degradation and weakening of the fibrous cap ultimately promoting a vulnerable plaque phenotype and plaque rupture.

Figure 6. Polymorphonuclear neutrophilic leukocyte (PMN) depletion in interferon regulatory factor 8-deficient (IRF8−/−) bone marrow (BM)-recipient low-density lipoprotein receptor-deficient (LDLR−/−) mice prevents exacerbated atherosclerotic lesion formation. A and B, Quantification of Oil Red O lipid depositions in the aorta (A) and aortic roots (B) of LDLR−/− mice reconstituted with IRF8+/+ (n=5) and IRF8−/− BM (n=6), and LDLR−/− mice reconstituted with IRF8−/− BM (n=6) treated with anti-PMN antibody for 5 weeks of high-fat diet. C, Quantification of macrophages (n=5–6), PMN (n=5–6), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL+) apoptotic cells (n=5–6), and necrotic core sizes (n=5–6) in aortic root plaques. *P<0.05, **P<0.01. MOMA-2 indicates monocyte/macrophage marker.
ApoE−/− or LDLR−/− mice transplanted with IRF8−/− BM displayed a marked expansion of PMN in peripheral blood and atherosclerotic lesions. The accumulation of PMN was also found to be increased in air pouches of IRF8−/− mice, and to be proportional to circulating PMN counts. Because no differences in PMN numbers were observed in the air pouch or the peritoneum in IRF8−/− compared with IRF8−/− mice under physiological conditions or without inflammation, these findings suggest that peripheral PMN numbers determine the rate of extravasation in the context of inflammation and atherosclerosis. The rapid PMN mobilization and leukocytosis under inflammatory conditions might thus constitute a universal mechanism imposing an enhanced PMN influx at sites of inflammation. In the context of myeloproliferative diseases, however, this might implicate an acceleration of atheroprotein.

PMN are short-lived phagocytes undergoing rapid apoptosis after emigration. The rate of apoptosis of blood or emigrated PMN was not different in IRF8−/− compared with control mice. However, likely due to their enhanced accumulation, apoptotic PMN were detected in increased numbers in atherosclerotic plaques. The impaired clearance of apoptotic cells and an overload of reduced phagocytic capacities of macrophages with dying PMN in mice carrying IRF8−/− BM might in consequence lead to necrosis, secondary inflammation, and necrotic core formation. Support for this notion stems from the prevention of enlarged necrotic core formation in LDLR−/− and apoE−/− mice reconstituted with IRF8−/− and IRF8−/−apoE−/− BM, respectively, and depleted of PMN. Depletion of PMN abolishes continuous neutrophil influx. However, such treatment can also reduce neutrophil counts in BM (Oliver Soehnlein, unpublished observations, 2011), suggesting that PMN depletion may also directly target tissue-resident neutrophils within lesions.

IRF8 plays a crucial role in the development and functionality of dendritic cells. Mice lacking dendritic cells due to a constitutive cell-specific expression of a suicide gene develop a myeloproliferative disorder, likely attributable to increased FMS-like tyrosine kinase 3 ligand serum levels. Notably, FMS-like tyrosine kinase 3 ligand serum levels are similarly increased in IRF8−/− mice. Although the impact of dendritic cells on atherosclerosis remains to be fully elucidated, an impaired functionality of dendritic cells in IRF8−/− mice may thus contribute to myeloproliferation and atherogenesis.

Taken together, our data for the first time show that the expansion of PMN in myeloproliferative syndromes or related conditions may give rise to enhanced atherosclerosis. In mice with IRF8−/− BM, functionally intact PMN may initiate vascular damage and atherosclerosis. In addition, their continuous influx might exacerbate inflammation in the local plaque environment aggravating disease progression and necrotic core formation in alliance with a defect in macrophage clearance of apoptotic cells and anti-inflammatory functions. However, functional impairments in monocytes/macrophages and dendritic cells or other cell types in IRF8−/− mice may additionally contribute to exacerbated lesion formation. Further clinical studies are warranted to scrutinize the possibility of an enhanced susceptibility to atherosclerosis in patients with myeloproliferative disorders.
Hematopoietic Interferon Regulatory Factor 8-Deficiency Accelerates Atherosclerosis in Mice

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SUPPLEMENT MATERIAL

Detailed Methods

Mouse models of atherosclerosis

All mice were provided with water and diet *ad libitum*, and were exposed to constant light-dark cycles, temperature and humidity. 6-8 weeks old, female recipient *Apoe*<sup>-/-</sup> or *Ldlr*<sup>-/-</sup> mice (obtained from Charles River Laboratories, C57BL/6 background, backcrossed 10 times into C57/Bl/6 background) were transplanted with bone marrow cells from either *Irf8*<sup>+/+</sup> or *Irf8*<sup>-/-</sup> mice<sup>1</sup> (C57BL/6 background, kindly provided by Prof. Ivan Horak, Research Institute for Molecular Pharmacology, University of Berlin) by tail vein injection 24 hours after an ablate dose of whole body irradiation (2×6.5 Gy). After 4 weeks of reconstitution, *Apoe*<sup>-/-</sup> and *Ldlr*<sup>-/-</sup> mice were fed an atherogenic diet containing 21% fat and 0.15 % cholesterol (Altromin) for 12 or 5 weeks, respectively. In addition, *Irf8*<sup>+/+</sup> and *Irf8*<sup>-/-</sup> mice were fed a high fat diet for 12 weeks. PMN depletion in *Ldlr*<sup>-/-</sup> mice was performed by intaperitoneal injections of mAb RB6-8C5 (100 μg/mouse, BioXCell) over 5 weeks every second day. Efficiency and specificity of PMN depletion was confirmed as shown before<sup>2</sup> (<sup>Supplementary Figure VII</sup>). Differential blood cell countswere determined by routine laboratory assays (Department of Clinical Chemistry, University Hospital Aachen). Cholesterol and triglyceride levels were analyzed by EnzyChrom<sup>™</sup> assays from BioTrend according to the manufacturer’s instructions. Briefly, triglycerides were assayed by hydrolysis and glycerol determination (EnzyChrom<sup>™</sup> Triglyceride Assay Kit). Total cholesterol was quantified by cholesterol ester hydrolysis and subsequent oxidation; HDL and LDL/VLDL were separated based on a polyethylene glycol precipitation method, and subjected to cholesterol ester hydrolysis and oxidation (EnzyChrom<sup>™</sup> AF HDL and LDL/VLDL assay kit); the color intensity of the
reaction product was then directly proportional to cholesterol. Animal experiments were approved by local authorities and complied with German animal protection law.

**Atherosclerotic lesion quantification and immunohistochemical analysis**

The extent of atherosclerosis was assessed in aortic roots and thoracoabdominal aortas by staining for lipid depositions with oil-red-O as described, quantified by computerized image analysis (Diskus Software) and Leica Qwin Imaging software (Leica Ltd.). In brief, atherosclerotic lesions were quantified in 5 μm transversal sections through the heart and aortic roots and the average calculated from 3-10 sections. The thoracoabdominal aorta was opened longitudinally, and the plaque area calculated as percentage of total thoracoabdominal aortic surface. The relative content of macrophages, T cells, SMCs, and PMN was determined by mAb staining for MOMA-2 (MCA519), CD3 (MCA1477, Serotec), smoothelin (N-15, Santa Cruz) and Ly6G (1A8, BD Biosciences), respectively. In addition, staining for Icam-1 (3E2, BD Biosciences), Myeloperoxidase (RB-373-AO, Neomarkers) and MMP9 (M17, Santa Cruz) were performed using the specific mAbs as indicated. Stainings were visualized by fluorescein isothiocyanate- or cyanine-3-conjugated secondary antibodies (Jackson ImmunoResearch). Appropriate IgG antibodies served as isotype controls. Apoptotic nuclei were detected by terminal deoxynucleotidyl nick-end labeling (TUNEL-kit, Roche). Nuclei were counterstained by 4',6-Diamidino-2-phenylindol (DAPI). Collagen was stained using Sirius red (Polysciences). Images were recorded with a Leica DMLB fluorescence microscope and CCD camera.

**Flow cytometry and cell sorting**

Whole blood obtained from the retro-orbital plexus of mice was EDTA-buffered and subjected to red-cell lysis (Pharmlyse BD Biosciences). Blood, bone marrow cells and peritoneal macrophages harvested 4 days after thioglycollate-induced peritonitis were
suspended in HBSS containing 0.3 mM EDTA and 0.1 % BSA and stained with antibodies to CD45, CD3, Gr1, CD11b, CD11c, CD36, CD40 (eBioscience), CD45, CD19, MHC II (BD Biosciences), Ly6G, F4/80, CD14 and CD16 (Bio Legend). Samples were analyzed and cells sorted after appropriate fluorescence compensation and gating strategies using a FACSCanto-II or FACSARia and FACSDiva software (BD Biosciences) analyzed using FlowJo software version 7.2.4 (Treestar). T cells were identified by staining for CD45 and CD3, B cells by CD45 and CD19, and DCs by CD45, CD11c, and MHC II. Intracellular staining of IFNγ and IL6 was done with antibodies and isotype controls from ebioscience.

Subcutaneous air pouch

A subcutaneous air pouch was induced as previously described. In brief, mice (10 weeks of age) were injected at day 0 and 4 with 5 ml sterile air subcutaneously in the back. At day 7, 1 ml sterile PBS containing PAF (1µM, Biomol) was injected into the pouch. 4 hours later the pouch was lavaged with 5 ml of ice-cold HBSS containing 0.3 mM EDTA and 0.1 % BSA. Thereafter, cells in the lavage fluid were counted manually using a Neubauer chamber and processed for FACS analysis as described above.

Analysis of MPO and MMP-9 activity and reactive oxygen species formation

MPO activity within the air pouch lavage fluid was measured by spectrophotometry (Tecan Spectrafluor Plus) using enzyme-specific substrates as described. MMP-9 activity was quantified using the SensoLyte MMP-9 assay kit (Anaspec), based on the proteolytic cleavage of a specific substrate. The fluorescent product was measured at 520 nm using a Tecan Spectrafluor Plus. For the detection of ROS formation, PMN from Irf8+/+ or Irf8−/− mice were seeded in 96-well plates and loaded with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes) in PBS at a final concentration of 10 µM for 30 min at 37°C.
Fluorescence was measured before and every 4 minutes after exposure to TNF-α (10 ng/ml, Peprotech), PMA (10 ng/ml, both Sigma), or vehicle (PBS) using a Tecan Spectrafluor Plus.

**Apoptosis and cell cycle assays**

PMN sorted from the air pouch lavage, blood and bone marrow were cultured in RPMI1640 (Gibco) containing 10 % FCS (Gibco) and 1% Penicillin/Streptomycin (Gibco). Cells were analyzed at 0, 12, 36, 60 and 84 hours of culture by staining with Annexin V in staining buffer (BD Bioscience) or propidium iodide (10 µg/ml, Sigma) in PBS buffer containing 0.25 % TritonX-100 (Sigma) and RNase A (4 µg/ml, GenScript) following fixation with 70 % ethanol (Sigma). Apoptosis and cell cycle were assessed by FACS analysis using a FACS Calibur (BD Biosciences).

**Peritonitis**

*Irf8*+/+ or *Irf8*−/− mice were left untreated or injected intraperitoneally with thioglycollate (Sigma, 3%) and peritoneal leukocytes were lavaged after 4 days as described3, 5.

**Phagocytosis assays**

Peritoneal macrophages harvested 4 days after thioglycollate-induced peritonitis were seeded into 96-well plates and incubated with di-labeled oxLDL (10 µg/ml) generated as described6 or calcein (Invitrogen)-labeled apoptotic PMN (1:1 ratio) for 1 hour. PMN were rendered apoptotic by serum deprivation over night and apoptosis was confirmed by Annexin V binding (>95%). Efferocytosis assays with apoptotic neutrophils and peritoneal macrophages were performed as described by Dr. Tabas (http://www.cumc.columbia.edu/dept/medicine/tabas_site/protocols/in%20vitro%20Effero%20Assay%20Protocol.pdf). Efferocytosis of apoptotic PMN and phagocytosis of oxLDL were analyzed by FACS.
Cytokine measurements and ELISA

Macrophages cultured in RPMI-1640 supplemented with 10% FCS and 1% Penicillin/Streptomycin were stimulated overnight with dead PMN, and IL-10 concentrations in cell-free supernatants were measured with a R&D mouse IL-10 Duo-Set ELISA Kit according to the manufacturer’s protocol.

Statistics

Data are represented as mean ± SD, and were analyzed by Student’s t-test, ANOVA with Tukey’s multiple comparison test, non-parametric Mann-Whitney test, or Kruskal-Wallis test with Dunn’s post-hoc test (Prism 4.0 software, GraphPad), as appropriate. Data for ROS measurement were analyzed with two-way repeated measures analysis of variance (ANOVA; treatment × time) followed by planned comparisons. p<0.05 was considered to be statistically significant.

Supplemental References


Supplemental Tables, Figures and Figure Legends

Supplemental Table I. Lipid levels

<table>
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<th>BM</th>
<th>Recipient</th>
<th>total cholesterol [mg/dL]</th>
<th>LDL/VLDL [mg/dL]</th>
<th>HDL [mg/dL]</th>
<th>triglycerides [mg/dL]</th>
<th>Number of mice [n]</th>
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<tr>
<td>1Ir8+/−</td>
<td>Apo−/−</td>
<td>211.0 ± 33.4</td>
<td>93.1 ± 10.1</td>
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<td>266.0 ± 34.0</td>
<td>126.7 ± 15.2</td>
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<td>252.0 ± 38.4</td>
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<td>1Ir8+/−</td>
<td>d-Ab</td>
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<td>98.6 ± 8.5</td>
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<tr>
<td>1Ir8+/−</td>
<td>Apo+/−</td>
<td>615.4 ± 15.4</td>
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<td>200.4 ± 21.8</td>
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<td>633.2 ± 28.3</td>
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</table>

LDL, low-density lipoprotein
VLDL, very low-density lipoprotein
HDL, high-density lipoprotein
d-Ab = PMN depleting antibody

Data represent mean ± SEM; no significant differences were observed between groups of mice in same experiments.
Supplemental Figure I

Supplemental Figure I. Representative images showing the quantification of extracellular versus intracellular lipid accumulations.
Supplemental Figure II

Supplemental Figure II. Increased atherosclerotic lesion formation in \textit{Apoe}^{−/−} mice with \textit{Irf8}^{+/−} \textit{Apoe}^{−/−} BM. (a,b) Quantification of oil-red-O$^+$ lipid depositions in the aortic root (a) and aorta (b) of \textit{Apoe}^{−/−} mice reconstituted with \textit{Irf8}^{+/+} \textit{Apoe}^{−/−} (n= 10) and \textit{Irf8}^{−/−} \textit{Apoe}^{−/−} BM (n=8-9); representative images are shown. (c) Quantification of acellular necrotic cores in \textit{Apoe}^{−/−} mice reconstituted with \textit{Irf8}^{+/+} \textit{Apoe}^{−/−} (n= 10) and \textit{Irf8}^{−/−} \textit{Apoe}^{−/−} BM (n= 7). *p<0.05, **p<0.01, ***p<0.001.
Supplemental Figure III. MPO and MMP-9-expression and collagen content in atherosclerotic lesions. (a,b,c) Aortic root plaques of Apoe<sup>-/-</sup> mice reconstituted with Irf8<sup>+/+</sup> and Irf8<sup>-/-</sup> BM were analyzed after 12 weeks of high fat diet. Quantification and representative images of staining for MMP-9 (a, n=4-5) and MPO<sup>+</sup> cells (b, n=4 each); cell nuclei are stained by DAPI. (c) Quantification and representative images of Sirius red staining (n=5 each). *p<0.05, **p<0.01.
Supplemental Figure IV. Icam-1 expression in atherosclerotic lesions. Quantification of Icam-1 expression in aortic root lesions of *Apoe*<sup>-/-</sup> mice reconstituted with *Irf8*<sup>+/+</sup> and *Irf8*<sup>-/-</sup> BM; representative images are shown (n=6 each). *p*<0.05.
Supplemental Figure V. Irf8-deletion does not affect macrophage apoptosis and proliferation. Apoptosis (left panel) and proliferation (right panel) in peritoneal macrophages harvested from Irf8+/+ or Irf8−/− mice, and after exposure to oxLDL (100µg/ml) at indicated time points, as assessed by determining Annexin V-binding capacities and cell cycle properties by flow cytometry, respectively (n=4 mice each).
Supplemental Figure VI

(a) Serum IL-10 concentrations in Apoe<sup>-/-</sup> mice reconstituted with Irf8<sup>-/-</sup> (n=5) and Irf8<sup>+/+</sup> BM (n=4) after 12 weeks of high fat diet (left panel), and in Ldlr<sup>-/-</sup> mice reconstituted with Irf8<sup>-/-</sup> (n=4) and Irf8<sup>+/+</sup> BM (n=5 each) after 5 weeks of high fat diet (right panel), as determined by ELISA.

(b) Relative frequencies of leukocyte subsets in the air pouch lavage fluid of Irf8<sup>+/+</sup> and Irf8<sup>-/-</sup> mice 4 hours after the injection of PAF (n=5-6 each), as assessed by flow cytometry.

Supplemental Figure VI. Cytokine production is impaired in Irf8<sup>-/-</sup> mice. (a) Serum IL-10 concentrations in Apoe<sup>-/-</sup> mice reconstituted with Irf8<sup>-/-</sup> (n=5) and Irf8<sup>+/+</sup> BM (n=4) after 12 weeks of high fat diet (left panel), and in Ldlr<sup>-/-</sup> mice reconstituted with Irf8<sup>-/-</sup> (n=4) and Irf8<sup>+/+</sup> BM (n=5 each) after 5 weeks of high fat diet (right panel), as determined by ELISA. (b) Relative frequencies of leukocyte subsets in the air pouch lavage fluid of Irf8<sup>+/+</sup> and Irf8<sup>-/-</sup> mice 4 hours after the injection of PAF (n=5-6 each), as assessed by flow cytometry.
Supplemental Figure VII. Neutrophils are efficiently depleted by injection of mAb RB6-8C5. *Irf8^{+/+}* (n=3) or *Irf8^{-/-}* mice (n=5) were injected i.p. with RB6-8C5 (100μg) and circulating neutrophil counts were assessed before (intact WBC) and 4 hours after injection PMN depletion.
Supplemental Figure VIII. PMN depletion in Irf8⁻/⁻ Apoe⁻/⁻ BM-recipient Apoe⁻/⁻ mice prevents exacerbated atherosclerosis. (a, b) Quantification of oil-red-O⁺ lipid depositions in the aorta (a) and aortic roots (b) of Irf8⁺/⁺ Apoe⁻/⁻ mice (n=10), and Irf8⁻/⁻ Apoe⁻/⁻, and Irf8⁻/⁻ Apoe⁻/⁻ BM-recipient Apoe⁻/⁻ mice treated with anti-PMN Ab for 4 weeks of high fat diet (n=4-6). (c) Quantification of necrotic core sizes and frequencies of lesional PMN (n=4-10). *p<0.05, **p<0.01.