**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> (Supplementary Figure VII).

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\(^3\), quantified by computerized image analysis (Diskus Software) and Leica Qwin Imagingsoftware (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\(^3\), \(^4\) 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 fluoresceinisothiocyanate- 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 counter-stained by 4',6-Diamidino-2-phenyldindol (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 (PharmlyseBD 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 FACS Diva 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 described2. 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 described2,5. 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 overnight 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 Tukeys 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

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<tr>
<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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<td>Irf8+/+</td>
<td>Apoe−/−</td>
<td>211.0 ± 33.4</td>
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<td>Irf8−/−</td>
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<td>266.0 ± 34.0</td>
<td>126.7 ± 15.2</td>
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<td>Ldlr−/−</td>
<td>252.0 ± 38.4</td>
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<tr>
<td>Irf8−/−</td>
<td>d-Ab</td>
<td>253.8 ± 23.9</td>
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<td>94.1 ± 4.8</td>
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<td>Apoe−/−</td>
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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 Apoe<sup>-/-</sup> mice with Irf8<sup>-/-</sup> Apoe<sup>-/-</sup> BM. (a,b) Quantification of oil-red-O<sup>+</sup> lipid depositions in the aortic root (a) and aorta (b) of Apoe<sup>-/-</sup> mice reconstituted with Irf8<sup>+/+</sup> Apoe<sup>-/-</sup> (n=10) and Irf8<sup>-/-</sup> Apoe<sup>-/-</sup> BM (n=8-9); representative images are shown. (c) Quantification of acellular necrotic cores in Apoe<sup>-/-</sup> mice reconstituted with Irf8<sup>+/+</sup> Apoe<sup>-/-</sup> (n=10) and Irf8<sup>-/-</sup> Apoe<sup>-/-</sup> 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). *<i>p</i><0.05, **<i>p</i><0.01.
Supplemental Figure IV. Icam-1 expression in atherosclerotic lesions. Quantification of Icam-1 expression in aortic root lesions of Apoe^{-/-} mice reconstituted with Irf8^{+/+} and Irf8^{-/-} 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\textsuperscript{+/+} or Irf8\textsuperscript{−/−} 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. Cytokine production is impaired in \textit{Irf8}^{-/-} mice. (a) Serum IL-10 concentrations in \textit{Apoe}^{-/-} mice reconstituted with \textit{Irf8}^{-/-} (n=5) and \textit{Irf8}^{+/+} BM (n=4) after 12 weeks of high fat diet (left panel), and in \textit{Ldlr}^{-/-} mice reconstituted with \textit{Irf8}^{-/-} (n=4) and \textit{Irf8}^{+/+} 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 \textit{Irf8}^{+/+} and \textit{Irf8}^{-/-} 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*<sup>+/−</sup>*Apoe<sup>−/−</sup> BM-recipient *Apoe<sup>−/−</sup> mice prevents exacerbated atherosclerosis. (a,b) Quantification of oil-red-O<sup>+</sup> lipid depositions in the aorta (a) and aortic roots (b) of *Irf8*<sup>+/−</sup>*Apoe<sup>−/−</sup> mice (n=10), and *Irf8*<sup>−/−</sup>*Apoe<sup>−/−</sup>, and *Irf8*<sup>−/−</sup>*Apoe<sup>−/−</sup> BM-recipient *Apoe<sup>−/−</sup> 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.