

**In adherence to the AHA Journals' implementation of the TOP Guidelines we state: The data that support the findings of this study are available from the corresponding author upon reasonable request.**

## Mice

*ChemR23<sup>el/e</sup>* mice were generated by Ozgene in C57/BL6-ES cells. All further breeding was done in C57/BL6 mice and all animals used in this study were on C57/BL6 background. Strategy for the generation of a *ChemR23* knock out/knock in reporter mouse expressing the enhanced Green fluorescent protein (eGFP) is depicted in **Figure 1 online-only Data Supplement**. *ChemR23<sup>el/e</sup>* mice were crossed with Apolipoprotein E deficient (*Apoe*<sup>-/-</sup>) mice to generate *ChemR23<sup>el/e</sup>Apoe*<sup>-/-</sup> mice (all on C57BL/6 background). C57BL/6 mice were purchased from Janvier Labs. The animals were bred in the local animal facility under SPF status and fed a normal laboratory diet (Sniff V1534-300) before the start of the experimental diet. For atherosclerosis studies, mice were fed a western-type diet (WD) containing 21% fat and 0.15-0.2% cholesterol (Altromin 132010, Ssniff TD88137), starting at 8-10 weeks of age for 4 or 12 weeks. Animals were sex and age matched also in adherence to the guidelines as described in the ATVB Council Statement (ATVB 2018; 38:292-303.). We have also confirmed that gender does not influence the directionality of our results specifically with respect to lesion size. Based on these findings we have either conducted our experiments in female mice (BMT recipients) or mixed gender (peritonitis, diet studies, adoptive transfer). All animal experiments were approved by the local ethical committee (Regierung von Oberbayern, Sachgebiet 54, Germany).

## Bone marrow transplantation

Bone marrow cells ( $3 \times 10^6$ /mouse) from *ChemR23<sup>el/e</sup>Apoe*<sup>-/-</sup> mice or from *Apoe*<sup>-/-</sup> littermate controls were flushed from femur and tibia cavities and subsequently administered to *Apoe*<sup>-/-</sup> recipient mice by lateral tail vein injection one day after a lethal dose of whole-body irradiation (2x 6.5 Gy). After four weeks of recovery, the mice were placed on WD for 6 weeks, as described above.

## Histology and immunofluorescence

Atherosclerotic lesion size was assessed by analyzing cryosections of the aortic root by staining for lipid depositions with Oil-Red-O. In brief, hearts with the aortic root were embedded in Tissue-Tek O.C.T. compound (Sakura) for cryosectioning. Oil-Red-O<sup>+</sup> atherosclerotic lesions were quantified in 4  $\mu$ m transverse sections and averages were calculated from 3-5 sections.

Aortic arches with the main branch points (brachiocephalic artery, left subclavian artery and left common carotid artery) were fixed with 4% paraformaldehyde and embedded in paraffin. Lesion size was quantified after Hematoxylin and Eosin (H&E)-staining of 5  $\mu$ m transverse sections and averages were calculated from 3-4 sections.

To assess the cellular composition or inflammation of atherosclerotic lesions, aortic root sections were stained overnight with a selection of the following antibodies: anti-Mac2 (Cedarline), anti-CD206 (LSBio, LS-B9805), anti-SMA (Dako and Sigma Aldrich, Clone 1A4), anti-SiglecH (eBioscience), anti-Cmklr1 (Bioss), anti-eGFP (abcam, ab65556), anti-ICAM1 (BD pharmingen, clone: 3E2), anti-CD31 (Dianova, dia-310, Clone SZ31). Nuclei were counter-stained by 4',6-Diamidino-2-phenylindol (DAPI). After incubation with a secondary FITC- or Cy3-conjugated antibody (Life Technologies) for 30 minutes at room temperature, the sections were embedded with VectaShield Hard Set Mounting Medium (Vector laboratories) and analyzed using a Leica DM4000B LED fluorescence microscope and charge-coupled device (CCD) camera. Blinded image analysis was performed using Diskus, Leica Qwin Imaging (Leica Lt.) or Image J software. For each mouse and staining, 2-3 root sections were analyzed and data were averaged.

Masson's Trichrome staining was used to visualize and quantify collagen within the plaques. Based on this, necrotic core size (defined as anucleated area) and the fibrous cap thickness (measured at the thinnest point of the cap) were assessed.

To visualize apoptotic cell content, ApopTag Red In Situ Apoptosis Detection Kit (Merck Millipore, S7165) was used. For this, cryosections were boiled in Citrate buffer for antigen retrieval and subsequent staining was carried out according to manufacturer's instructions. Reaction volumes were adjusted to 30  $\mu$ L per section.

### **Intravital microscopy**

Leukocyte adhesion to the carotid artery was analyzed using intravital microscopy. Briefly, the right jugular vein was cannulated with a catheter for antibody and dye injection. After exposure of the left carotid artery, antibodies (1  $\mu$ g) to CD11b (eBioscience), Ly6G (BioLegend) and Ly6C (eBioscience) were sequentially administered to label various leukocyte subsets. Recordings were made 3 min after injection of each antibody. Finally, rhodamine 6G (100  $\mu$ L, 0.1% solution) was injected to label all circulating leukocytes. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis, Olympus Cell-R software was used.

### **Laboratory blood parameters and Flow cytometry**

Whole blood obtained from *Apoe*<sup>-/-</sup> control or *ChemR23*<sup>ex/e</sup> *Apoe*<sup>-/-</sup> mice was collected in EDTA-buffer tubes. Selected blood parameters were determined using a Celltac Automated Hematology Analyzer (Nihon Kohden). Afterwards, samples were subjected to red-blood-cell lysis for further analysis using flow cytometry. Bone marrow cells were harvested by flushing femurs with Hank's Medium (Hanks' Balanced Salt Solution + 0.3 mmol/l EDTA + 0.1% BSA) (Gibco by life technologies). Spleen and lymph nodes were mechanically crushed and passed through a 30  $\mu$ m cell strainer (Cell-Trics, Partec) using Hank's Medium and to obtain single cell suspensions. Single cell suspensions were subsequently stained with different antibody cocktails and analyzed using a FACS Canto II, using the FACSDiva software (BD Biosciences). Cell populations were discriminated by the following antibody cocktail: anti-CD45, anti-CD115, anti-Gr1, anti-CD11b, anti-F4/80, anti-B220, anti-CD3, anti-SiglecH 440c, anti-CD11c and anti-MHC class II (All eBioscience). Cell populations and marker expression were gated as depicted below using the FlowJo analysis program (Treestar): neutrophils (CD45<sup>+</sup>CD115<sup>+</sup>Gr1<sup>high</sup>), monocytes (CD45<sup>+</sup>CD11b<sup>+</sup>CD115<sup>+</sup>), and macrophages (CD45<sup>+</sup>F4/80<sup>+</sup>), lymphocytes (CD45<sup>+</sup>CD3<sup>+</sup> and CD45<sup>+</sup>B220<sup>+</sup>), plasmacytoid dendritic cells (pDCs; CD45<sup>+</sup>B220<sup>+</sup>SiglecH<sup>+</sup>) and conventional dendritic cells (cDCs; CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>). Surface expression of specific antigens are analyzed using anti-Cmklr1 (Bioss) or endogenous eGFP signal in *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice, anti-CCR7 (eBioscience), anti-CD86 (eBioscience) and anti-CD301 (Biozym) antibodies. Apoptosis was evaluated by AnnexinV staining according to the manufacturer's instructions (Invitrogen by ThermoFisher Scientific).

### **Plasma lipid levels**

Cholesterol and triglyceride levels were analyzed using mouse EDTA-buffered plasma and quantified using enzymatic assays (c.f.a.s. cobas, Roche Diagnostics) according to the manufacturer's protocol.

### **Functional analysis of pDCs and aortic macrophages**

pDCs are isolated from bone marrow using FACS sorting (BD FACSAria III). Bone marrow was isolated and stained as described above. pDCs are defined as CD45<sup>+</sup>B220<sup>+</sup>SiglecH<sup>+</sup>. The endogenous eGFP signal (ChemR23) was measured in the FITC channel. After cell sorting, pDCs were cultured in 24 well flat-bottom plates (10<sup>5</sup> cells/well) (Corning Costar by Sigma-Aldrich/Merck) in RPMI-1640 medium supplemented with L-Glutamine (Gibco by Life technologies) and 1% Penicillin/Streptomycin with or without 5  $\mu$ g/ml CpG oligodeoxynucleotides (ODN 1585, InvivoGen). After the incubation of pDCs for 12 hours

at 37°C, the supernatants were used for IFN- $\alpha$  ELISA and co-stimulatory molecule upregulation was measured by flow cytometry.

Macrophages are isolated from the aortic tree or aorta using FACS sorting. For this, tissues are lysed in 300  $\mu$ L media using 15  $\mu$ L Liberase at 37°C for 30 min. Subsequently, single cells are prepared and stained as described above. Macrophages are defined as CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>. The endogenous eGFP signal (ChemR23) was measured in the FITC channel. Isolated macrophages were further processed for PCR or flow cytometry analysis.

### **Relative quantification of gene expression with real time PCR**

The Pinpoint™ Slide RNA Isolation System (Zymo Research) was used to extract genetic material from aortic root sections. RNA from sorted macrophages was isolated with the Direct-zol™ RNA microprep kit from Zymo Research. Individual procedure steps can be found in the manufacturer's protocol. For aortic roots, three sections per mouse were combined to assess the gene expression of SiglecH. IL10, IL6, Arginase1 (Arg-1) mRNA expression was analyzed in sorted macrophages. Real time multiplex-PCR was performed using 10ng RNA per sample in a one-step-RT-PCR reaction mix (QuantiFast Multiplex RT-PCR Kit from Qiagen) in combination with TaqMan® Assays from ThermoFisher Scientific. Probes from ThermoFisher were IL6: Mm00446190\_m1, Cat.No. 4331182; IL10: Mm01288386\_m1, Cat.No. 4331182; Arginase 1: Mm00475988\_m1, Cat.No.4331182, SiglecH: Mm00618627\_m1, Cat.No.4331182 and as a reference gene 18sRNA: Mm03928990\_g1 (Vic/primer limited). Real-time PCR assays were performed with Applied Biosystems 7900H machine, changes in gene expression were calculated with Delta-delta CT method.

### **ELISA**

Chemerin plasma levels were quantified from EDTA-plasma of full blood by ELISA using a commercially available kit (Quantikine ELISA Mouse Chemerin by R&D systems) following the manufacturer's protocol. IFN- $\alpha$  levels from pDC culture supernatants were analyzed according to the manufacturer's protocol with a VeriKine mouse Interferon Alpha ELISA Kit (pbl Assay Science). The final measurement of absorbance was carried out using a plate reader set to 450 nm with a correction factor of 550 nm.

### **Chemotaxis assay**

pDCs were isolated via FACS sorting (BD FACSAria III) as described above. The assay was carried out using 48 or 96-well microchemotaxis Boyden chambers (NeuroProbe or Corning) with polycarbonate membranes (5 $\mu$ m pores; NeuroProbe). The chemotactic factors recombinant mouse chemerin (R&D Systems), CXCL12 (PeproTech Inc.) and CCL19 (PeproTech) were prepared to a final concentration of 100 ng/ml or 200ng/ml (CCL19) in chemotaxis assay buffer (RPMI-1640 medium supplemented with 0.5% BSA). Controls were performed in the absence of chemoattractant in the lower wells. Cells were seeded into the upper wells (~1x10<sup>5</sup> cells/well) of the plate. Some cells were treated with CpG (5ug/ml) prior (30 minutes) and during transmigration for a period of 3h at 37°C, cells from the were retrieved and analyzed by FACS analysis.

### **Adoptive transfer**

pDCs are isolated from bone marrow from *Apoe*<sup>-/-</sup> control or *ChemR23<sup>e/e</sup>Apoe*<sup>-/-</sup> mice using FACS sorting. Bone marrow was isolated and stained as described above. pDCs are defined as CD45<sup>+</sup>B220<sup>+</sup>SiglecH<sup>+</sup>. Isolated pDCs are stained using cell tracker proliferation dye eF670 (Invitrogen by ThermoFisher Scientific). Recipient *Apoe*<sup>-/-</sup> mice are fed a WD for 10 weeks. Labelled pDCs are injected by lateral tail vein injection (~5x10<sup>5</sup> cells/recipient). After 24 hours, aortas are isolated and single cell suspensions are prepared and evaluated using flow cytometry as described before.

### **Bone marrow derived macrophages and cholesterol efflux/uptake**

Bone marrow was isolated from femurs and tibiae of C57BL/6 control or *ChemR23<sup>e/e</sup>* mice. Cells were cultured in RPMI-1640 medium (GIBCO Invitrogen) supplemented with 10%

(vol/vol) heat inactivated fetal calf serum (Gibco by Life technologies), supplemented with L-Glutamine and 1% Penicillin/Streptomycin (Gibco by Life technologies) and 25ng/ml M-CSF (PeproTech Inc.) for 8-9 days to differentiate into bone marrow-derived macrophages (BMDMs).

Macrophages were incubated with 25µg/ml (ox)LDL or Dil-(ox)LDL (KB Kallen), unless stated otherwise, for 4 hours. Dil-(ox)LDL uptake was analyzed using FACS analysis, by measuring the Dil signal in the PE-channel.

ABCA1/ABCG1 expression was analyzed using FACS analysis, using anti-ABCA (NOVUS, NB400-105AF405) and anti-ABCG1 (GeneTex, N-terminal).

For the cholesterol efflux assay, cultured BMDMs were replated at  $0.5 \times 10^6$  cells per well in a 24-well plate and allowed to adhere overnight in culture medium. The next day the culture medium was replaced by culture medium containing tritium ( $^3\text{H}$ )-cholesterol (1 µCi/well; PerkinElmer, Waltham, MA, USA) and oxLDL (50 µg/ml, Biotrend) for 24 h. After loading the cells were equilibrated for 2 h in RPMI medium containing 0.2% BSA (both 2 Life Technologies). The equilibrated BMDM were subsequently incubated for 6 h with RPMI medium containing 0.2% BSA and ApoA1 (15 µg/ml, Sigma Aldrich) or HDL (50 µg/ml, Sigma Aldrich) or received no further treatment. The medium was removed, collected, and the cells were lysed at 37°C with 0.3 M sodium hydroxide (NaOH) solution for 15 min. The cell lysate was collected and both, the lysate and the supernatant were transferred to scintillation medium (Zinsser Analytic, Frankfurt, Germany) and radioactivity measured with a scintillator (PerkinElmer). Counts from cellular lysate added with counts from supernatant represent total cholesterol uptake whereas counts only for supernatant represent cholesterol efflux.

### **Efferocytosis assay**

BMDMs from C57BL/6 control or *ChemR23<sup>e/e</sup>* mice were differentiated as described above and replated at  $0.5 \times 10^6$  cells per well in a 12-well plate and allowed to adhere overnight in culture medium. Prior to the efferocytosis assay, macrophages were incubated with or without oxLDL (25µg/ml, KB Kallen) for 24 hours. Seminal vesicle epithelial cells (SVECs) were used as prey cells and made apoptotic by incubating the cells with staurosporin (0.3µM, Sigma Aldrich) for 16 hours. Subsequently, apoptotic SVECs are labelled with pH-sensitive pHrodo dye according to manufacturer's protocol (ThermoFisher Scientific). Labelled apoptotic SVECs are then added in different ratio's based on cell counts to the BMDMs and incubated for 3 hours under normal culture conditions. Finally, cells are harvested and used for flow cytometry analysis.

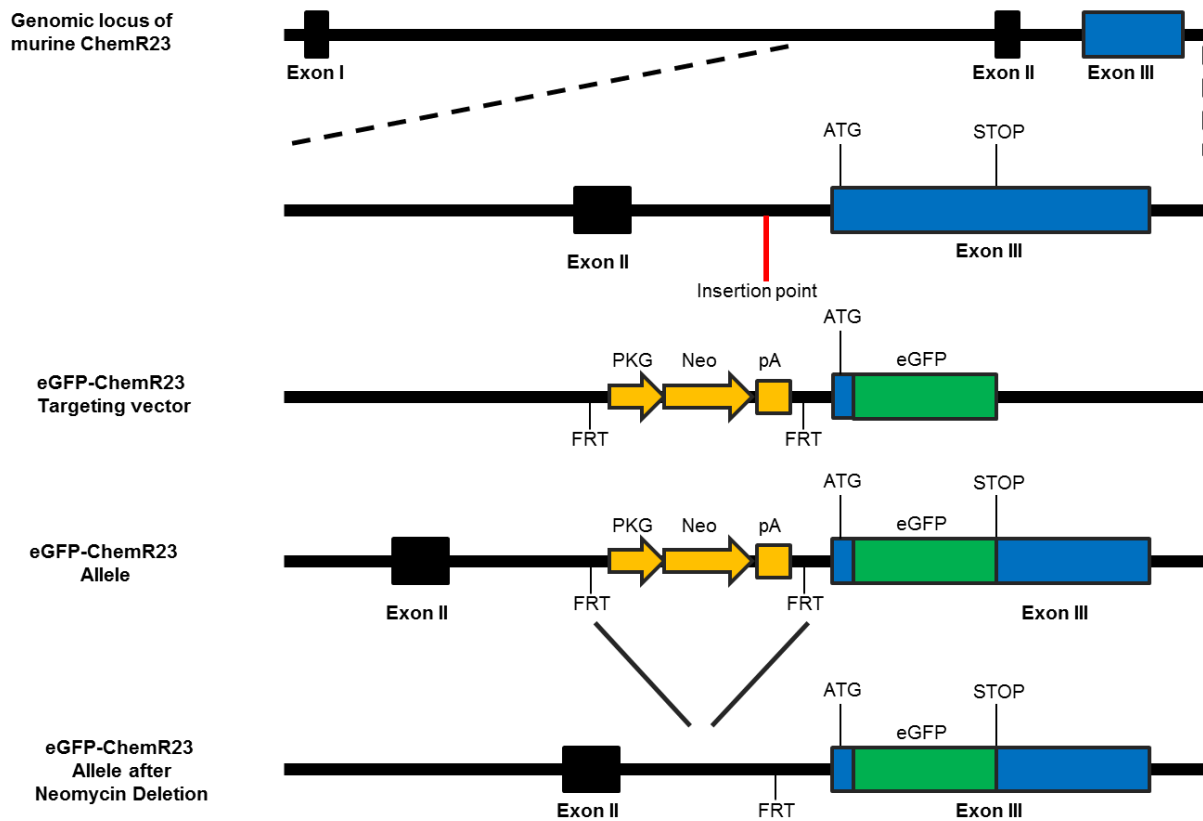
### **Peritonitis model**

In order to induce peritoneal inflammation, mice are injected i.p. with TNFα (50ng, PeproTech Inc.), chemerin (5µg, R&D Systems) or PBS as control. After 4 hours mice are euthanized and peritoneal lavages are collected by flushing the peritoneal cavity with 5ml ice-cold PBS. Isolated cells are subsequently used for flow cytometry analysis.

### **Statistics**

All data are expressed as mean±SEM, as indicated. Statistical calculations were performed using the up-to-date version of GraphPadPrism (GraphPad Software Inc.). After verifying normal distribution via D'Agostino-Pearson omnibus normality test, unpaired Student's t-test with Welch's correction, Mann-Whitney test, One-way ANOVA or Kruskal-Wallis with Dunnett's or Dunn's multiple comparisons or Two-way ANOVA with Sidak's multiple comparisons test were used, as appropriate. P-values <0.05 were considered as being statistically significant.

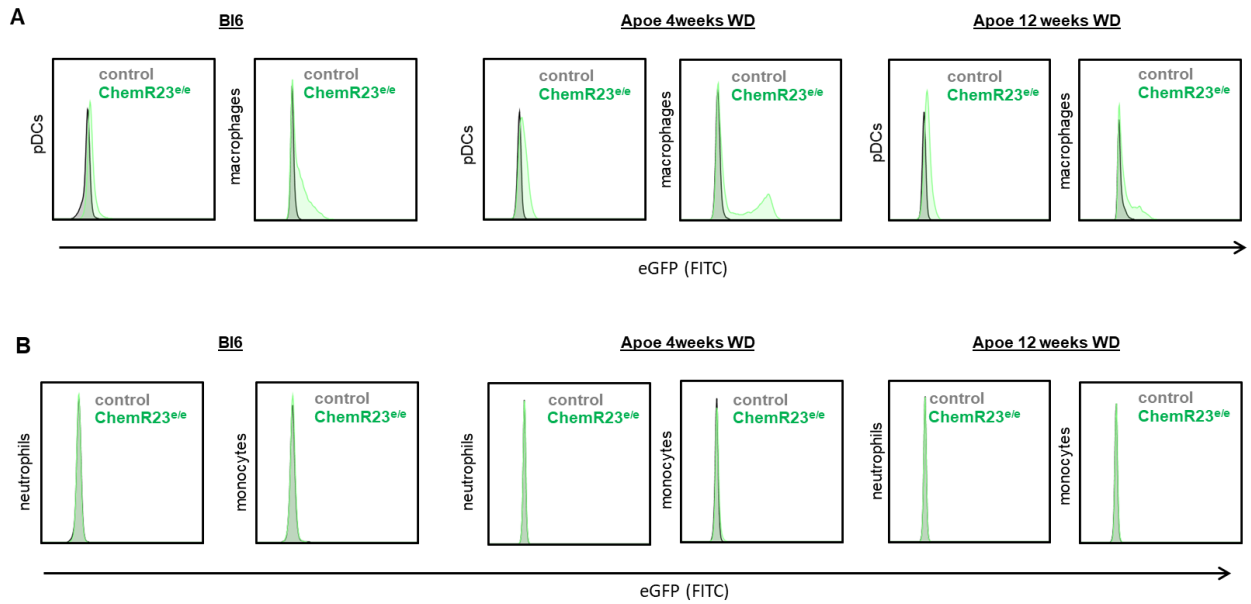
## Van der Vorst et al. online-only Data Supplement



### Supplemental Figure I.

#### Strategy for the generation of a ChemR23 *knock out/knock in* reporter mouse expressing the *enhanced Green fluorescent protein* (eGFP).

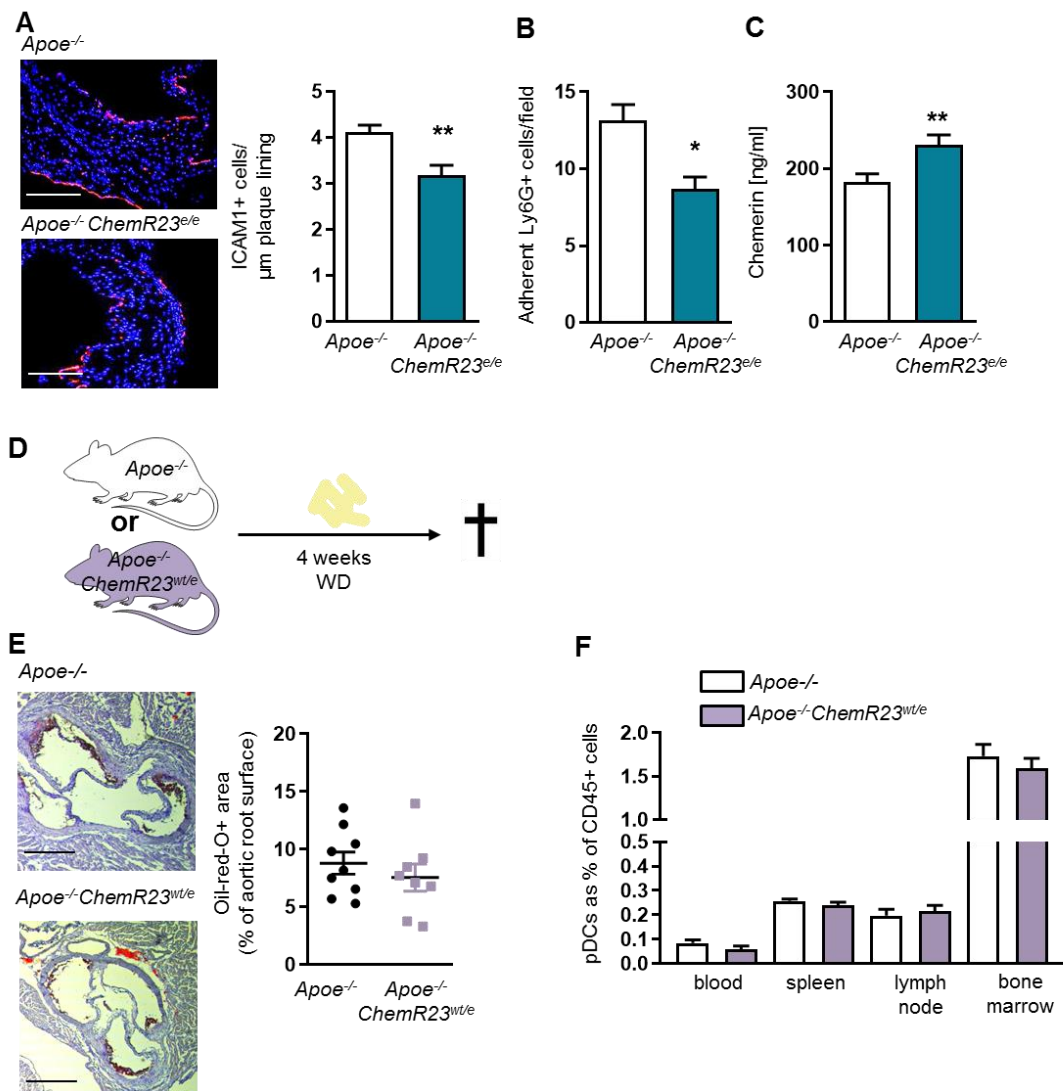
The gene for ChemR23 (*Cmklr1*) consists of 3 exons and is located on the reverse strand of chromosome 5. To generate the knock out/knock in, the entire coding sequence (ATG – STOP) is replaced by an eGFP open reading frame (Green bar). Additionally, at the insertion point, a neomycin selection cassette (FRT\_PKG\_Neo\_pA\_FRT) is inserted. The PKG\_Neo cassette is shown in yellow. Finally, the PKG\_Neo cassette is excised by FlpE-mediated recombination. This should not interfere with transcription through the locus. Transcripts should be spliced from non-coding exons 1 and 2 to exon 3, thereby enabling expression of eGFP.



### Supplemental Figure II.

**ChemR23 is particularly expressed on murine pDCs and macrophages.**

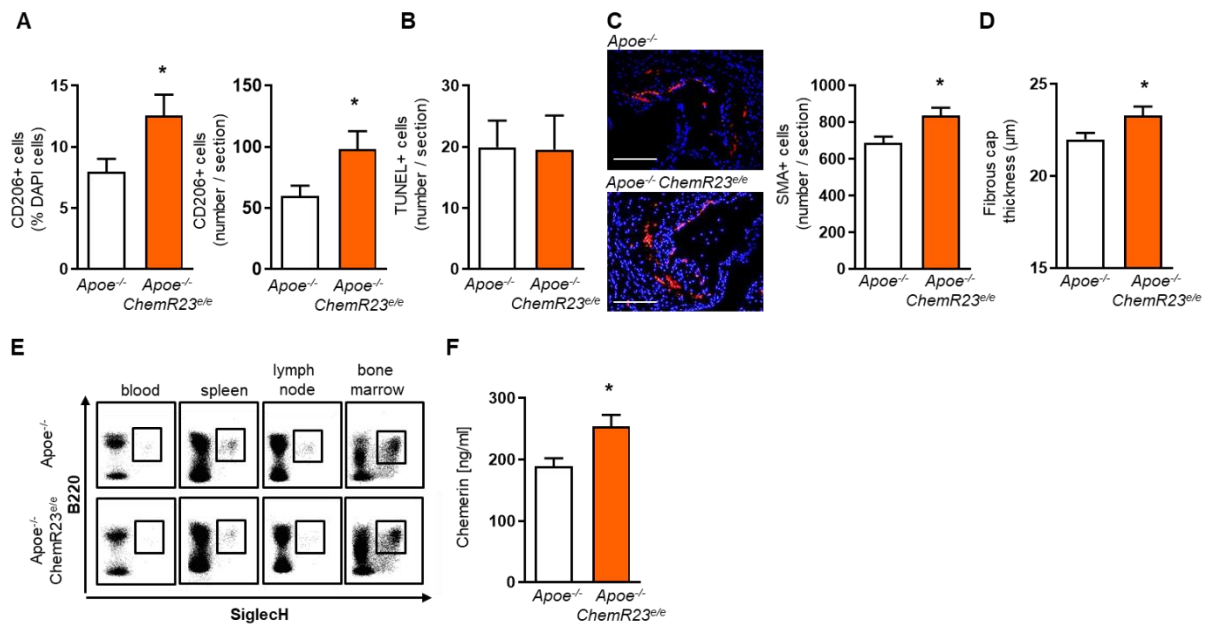
**(A-B)** Graphs show representative histograms of *ChemR23<sup>e/e</sup>* mice and controls (either *C57BL/6* or *Apoe<sup>-/-</sup>*, representative plots from n=3), pre-gating CD45, demonstrating ChemR23 expression (endogenous eGFP signal by eGFP knock-in in the *ChemR23* locus) on splenic pDCs and macrophages **(A)** but no expression on blood monocytes and neutrophils **(B)**.



**Supplemental Figure III.**

**ChemR23-deficiency reduces endothelial activation but increases chemerin levels after 4 weeks western type diet (WD), heterozygous ChemR23-deficiency does not affect atherosclerosis.**

(A) Representative picture and quantification of ICAM staining on aortic roots of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>e/e</sup> mice after 4 weeks WD (n=12-15). Scale bar = 250 μm; (B) Quantification of intravital microscopy of neutrophil (Ly6G+ cells) adhesion to TNFα-stimulated carotid arteries of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>e/e</sup> mice after 4 weeks WD (n=6 per group); (C) Plasma chemerin levels, measured using ELISA, of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>e/e</sup> mice after 4 weeks WD (n=18-20); (D) Experimental scheme of 4 weeks WD; (E) Representative pictures and quantification of lesion area measured after Oil-Red-O staining for lipid deposits in the aortic root of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>wt/e</sup> mice after 4 weeks WD (n=8-9). Scale bar = 500 μm; (F) Quantification of pDCs in different organs of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>wt/e</sup> mice (CD45+B220+SiglecH+), after 4 weeks WD; Data represent mean±SEM. \*P<0.05; \*\*P<0.01, as analyzed by Student's t-test with Welch correction or Mann-Whitney test, as appropriate.

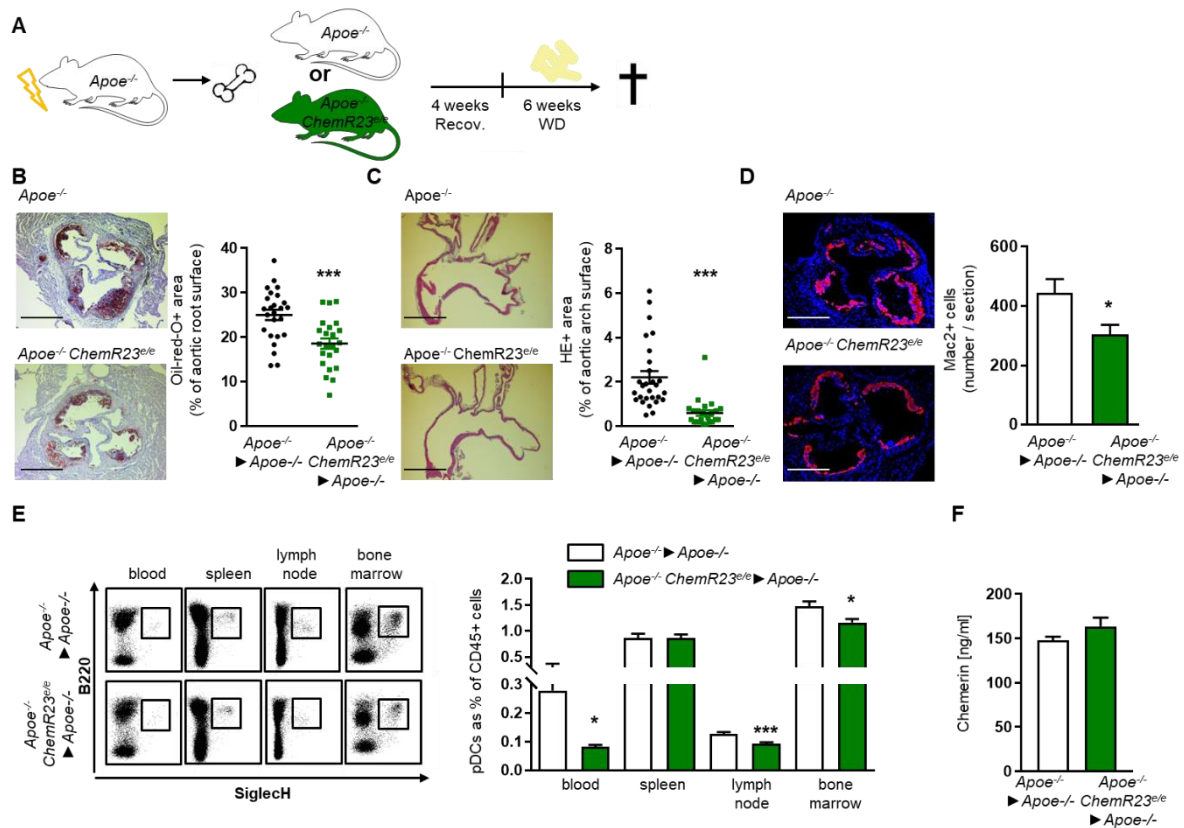


**Supplemental Figure IV.**

**ChemR23-deficiency enhances the number CD206<sup>+</sup> cells and smooth muscle cells (SMC) and increases chemerin levels after 12 weeks western-type diet (WD).**

(A) Quantification of CD206<sup>+</sup> macrophages (depicted as CD206<sup>+</sup> cells as % of DAPI<sup>+</sup> and as CD206<sup>+</sup> cells per section) in aortic root lesions of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice after 12 weeks WD (n=11-16) by immunohistological analysis. (B) Quantification of apoptotic cell numbers (depicted as TUNEL<sup>+</sup> nuclei per section) and quantified after TUNEL staining in aortic root lesions of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice 12 weeks WD (n=10). (C) Representative pictures and quantification of SMC content in aortic root lesions of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice, as quantified after SMA staining after 12 weeks WD (n=8-10). Scale bar = 250 μm; (D) Quantification of fibrous cap thickness, determined by measuring the cap thickness at the thinnest point in aortic root lesions of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice after 12 weeks WD (n=12-13); (E) Representative dot plots of pDCs in different organs as analyzed by flow cytometry, pre-gating CD45, of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice after 12 weeks WD; (F) Plasma chemerin levels, measured using ELISA, in *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup> mice after 12 weeks WD (n=17-20). Data represent mean±SEM. \**P*<0.05, as analyzed by Student's *t*-test with Welch correction or Mann-Whitney test, as appropriate.

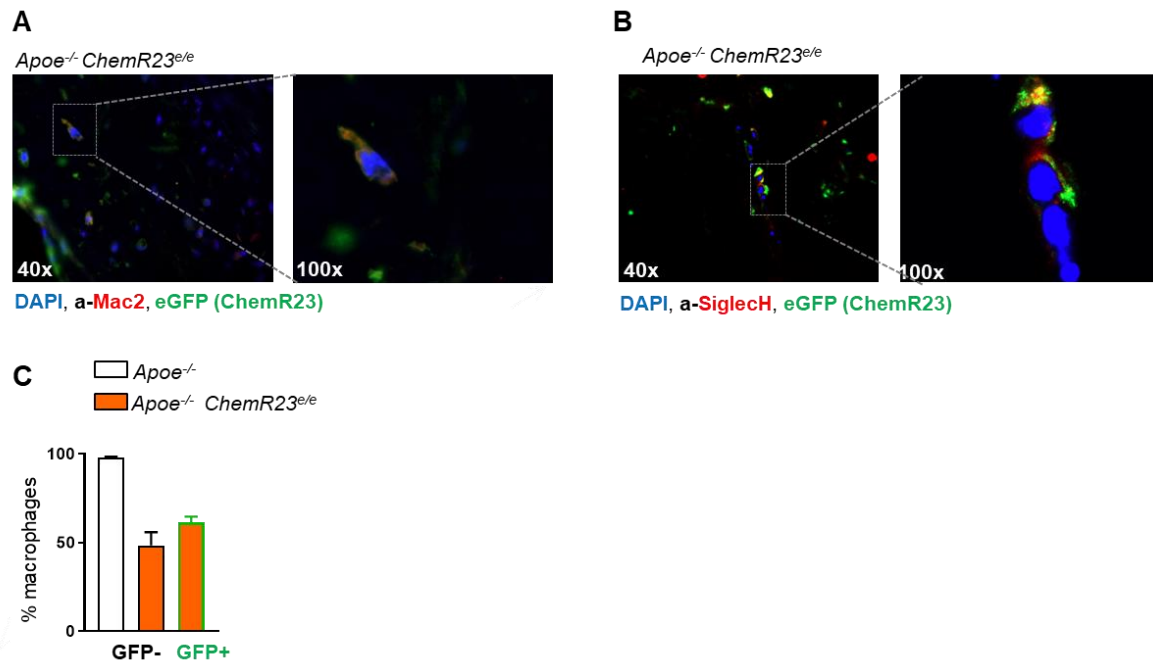




**Supplemental Figure V.**

**Hematopoietic ChemR23-deficiency reduces atherosclerotic plaque size after 6 weeks western-type diet (WD).**

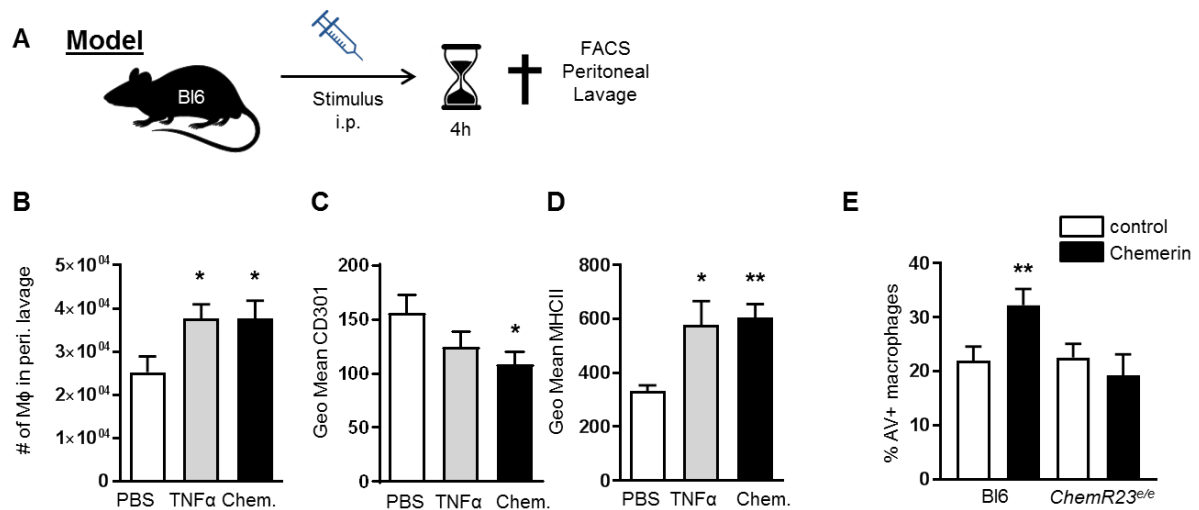
**(A)** Experimental scheme of bone marrow transplantation (BMT), followed by 4 weeks recovery and 6 weeks WD; **(B)** Representative pictures and quantification of lesion area measured after Oil-Red-O staining for lipid deposits in the aortic root of *Apoe*<sup>-/-</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> mice after BMT and 6 weeks WD. Scale bar = 500μm; **(C)** Representative images and quantification of atherosclerotic lesion size in aortic arches of *Apoe*<sup>-/-</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> mice, quantified using H&E staining after BMT and 6 weeks WD. Scale bar = 1mm; **(D)** Representative pictures and quantification of macrophage content in aortic root lesions of *Apoe*<sup>-/-</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> mice, as quantified after Mac2 staining after BMT and 6 weeks WD (n=11-16). Scale bar = 500μm; **(E)** Representative dot plots and quantification of pDCs by flow cytometric analysis in different organs, pre-gating CD45, of *Apoe*<sup>-/-</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> mice after BMT and 6 weeks WD (n=21-25); **(F)** Plasma chemerin levels, measured using ELISA, in *Apoe*<sup>-/-</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/e</sup>  $\rightarrow$  *Apoe*<sup>-/-</sup> mice after BMT and 6 weeks WD (n=10-18); Data represent mean±SEM. \**P*<0.05; \*\*\**P*<0.001, as analyzed by Student's t-test with Welch correction or Mann-Whitney test, as appropriate.



## Supplemental Figure VI.

### ChemR23 expressing macrophages and pDCs are present in atherosclerotic lesions.

**(A)** Double staining of Mac2 (Macrophages) and eGFP (ChemR23), with DAPI (Nuclei) counterstain in atherosclerotic lesions in aortic roots of *Apoe<sup>-/-</sup> ChemR23<sup>e/e</sup>* mice; **(B)** Double staining of SiglecH (pDCs) and eGFP (ChemR23), with DAPI (Nuclei) counterstain in atherosclerotic lesions in aortic roots of *Apoe<sup>-/-</sup> ChemR23<sup>e/e</sup>* mice; **(C)** Analysis of lesional macrophage content in aortic roots of *Apoe<sup>-/-</sup>* and *Apoe<sup>-/-</sup> ChemR23<sup>e/e</sup>* mice after 12 weeks WD, distinguishing between eGFP positive and negative cells as percentage of total macrophage cell count (n = 5 per group).

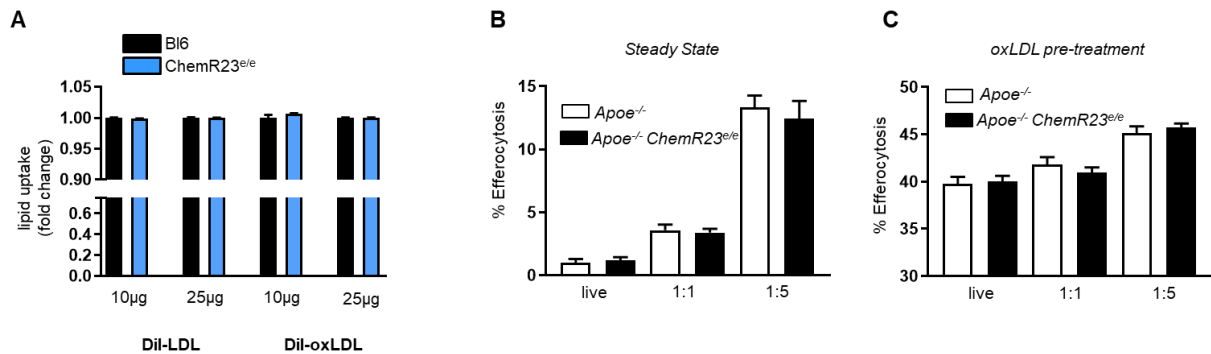


**Supplemental Figure VII.**

### **Chemerin induces a macrophage M1 phenotype and macrophage apoptosis**

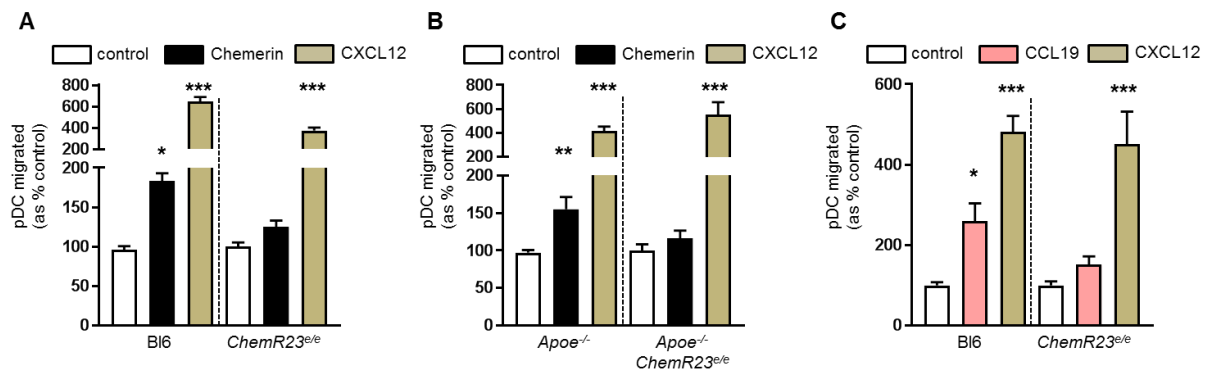
**(A)** Experimental scheme of peritonitis. *C57BL/6* mice are injected i.p. with the stimuli TNFα (50ng), chemerin (5μg) or PBS as control. After 4 hours, peritoneal lavage is taken and analyzed using FACS; **(B)** Quantification of macrophage (Mφ) numbers (defined as CD45<sup>+</sup>, F4/80<sup>+</sup> CD11b<sup>+</sup>) in the peritoneal lavage of *C57BL/6* mice (n=14-20); Surface expression of CD301 **(C)**; n=15-17) and MHCII **(D)**; n=16-17) on macrophages in the peritoneal lavage of *C57BL/6* mice, expressed as geometrical mean intensity (Geo Mean); **(E)** Splenocytes of *C57BL/6* (BL6) or *ChemR23<sup>Δ/e</sup>* mice were cultured in absence and presence of chemerin (100ng/ml) for 12 hour and annexin V (AV+) was quantified on splenic macrophages (markers) by flow cytometric analysis (n = 5 independent experiments); Data represent mean±SEM. \**P*<0.05; \*\**P*<0.01, as analyzed by One-way (B-D) or Two-way (E) ANOVA with Dunnett's or Sidak's multiple comparisons test, respectively. In B-D, significance is indicated compared to PBS control. In E, significance is indicated comparing control vs. chemerin treatment.

### Supplemental Figure VIII.



### ChemR23-deficiency does not affect macrophage lipid uptake or efferocytotic capacity.

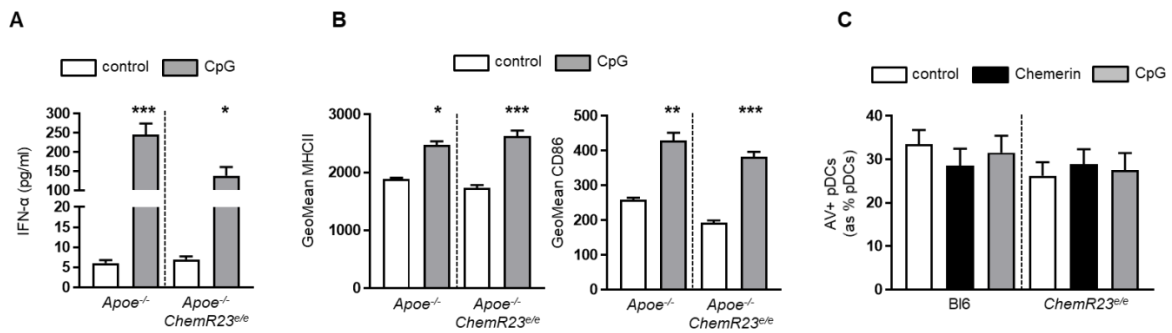
(A) Uptake of Dil-LDL or Dil-oxLDL by bone marrow derived macrophages (BMDMs) derived from *C57BL/6* (B16) or *ChemR23<sup>e/e</sup>* mice after 4 hours incubation. Uptake was analyzed by flow cytometry and normalized relative to B16 controls (n=4); (B, C) Comparison of the efferocytotic capacity of cultured BMDMs from *Apoe*<sup>-/-</sup> *ChemR23<sup>e/e</sup>* and *Apoe*<sup>-/-</sup> mice. In (C) BMDMs were pre-treated with oxLDL (25ug/ml) for 24 hours prior to assaying. Ratios refer to apoptotic prey cells (SVECs) which were stained with pH-sensitive pHrodo dye and added to BMDMs as live cells or in ratios 1:1 or 1:5 as indicated in the graphs. After 3 hours, flow cytometric analysis were performed (B, n=6, C, n=4). Data represent mean±SEM.



### Supplemental Figure IX.

#### Transmigration of *ex vivo* isolated ChemR23-deficient pDCs is diminished in response to chemerin, but not CXCL12.

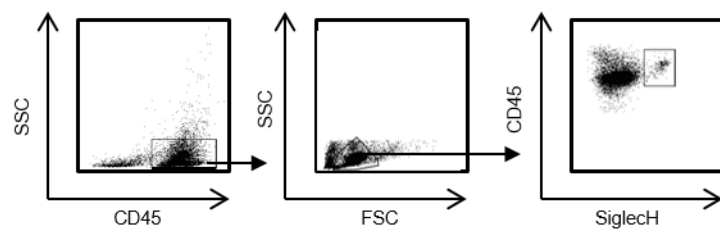
For *in vitro* analysis, pDCs are FACS-sorted from bone marrow cells of C57BL/6 (Bl6), ChemR23<sup>e/e</sup>, Apoe<sup>-/-</sup> or Apoe<sup>-/-</sup> ChemR23<sup>e/e</sup> mice and cultured in RPMI medium, containing 0.5% BSA. **(A, B)** Transmigration of pDCs towards chemerin (100ng/ml), CXCL12 (100ng/ml) or PBS (control) depicted as % migration of control (A: n= 9-11; B: n= 9-18). **(C)** Transmigration of pre-stimulated pDCs (CpG, 5μg/ml for 4h) towards CCL19 (200ng/ml), CXCL12 (100ng/ml) or PBS (control) depicted as % migration of control, n= 6-8. Migrated cells were quantified using FACS analysis; Data represent mean±SEM. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001 as analyzed by Two-way ANOVA with Sidak's multiple comparisons test compared to the respective control bar.



**Supplemental Figure X.**

### Impact of ChemR23-deficiency on pDC function and apoptosis.

For *in-vitro* analysis, pDCs are FACS-sorted from bone marrow cells of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>e/e</sup> mice and cultured in RPMI medium, containing 10% FCS and 1% Pen/Strep; **(A)** IFN $\alpha$  levels, measured by ELISA, in supernatants of cultured pDCs stimulated for 12 hours with 5 $\mu$ g/ml CpG or left untreated (control) (n=6); **(B)** Surface expression of MHCII and CD86, measured by FACS analysis, on cultured pDCs stimulated for 12 hours with 5 $\mu$ g/ml CpG or left untreated (control) (n=9); expressed as geometric mean intensity (Geo Mean); **(C)** Measurement of Annexin-V positivity (AV+) on cultured pDCs, using FACS analysis, pDCs were stimulated for 4 hours with 100ng/ml chemerin, 5 $\mu$ g/ml CpG or left untreated (control) (n=9-11); Data represent mean $\pm$ SEM. \**P*<0.05; \*\**P*<0.01, \*\*\**P*<0.0001 as analyzed by Two-way ANOVA with Sidak's multiple comparisons test compared to the respective control bar.



**Supplemental Figure XI.**

**Gating strategy for sorting pDCs from bone marrow.**

Representative gating strategy to identify and sort pDCs from the bone marrow.

**Supplemental Table I. Blood parameters of *Apoe*<sup>-/-</sup> vs. *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/ex</sup> mice after 4 weeks WD**

<u>4 weeks WD</u>	<i>Apoe</i> <sup>-/-</sup>	<i>Apoe</i> <sup>-/-</sup> <i>ChemR23</i> <sup>ex/ex</sup>	<i>P</i> -value
Cholesterol (mg/dL)	1162.0 ± 45.3	1190.0 ± 59.5	0.707
Triglycerides (mg/dL)	152.0 ± 10.8	118.8 ± 7.7	0.016
Body weight (g)	21.8 ± 0.3	22.6 ± 0.5	0.277
Leukocytes (cells x 10 <sup>6</sup> /ml blood)	3.13 ± 0.3	2.55 ± 0.3	0.184
Neutrophils (% of leukocytes)	14.67 ± 0.7	14.5 ± 0.7	0.932
Monocytes (% of leukocytes)	6.9 ± 0.3	8.0 ± 0.6	0.130
T cells (% of leukocytes)	25.3 ± 0.9	25.6 ± 1.0	0.388

**Table SI.** Listed are plasma cholesterol and triglycerides levels, body weight, total leukocytes counts and the percentage of neutrophils, monocytes and T cells in peripheral blood of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>ex/ex</sup> mice after 4 weeks of WD (n=15-25); Data represent mean±SEM, as analyzed by Student's t-test with Welch correction or Mann-Whitney test, as appropriate.



**Supplemental Table II. Blood parameters of *Apoe*<sup>-/-</sup> vs. *Apoe*<sup>-/-</sup>*ChemR23*<sup>o/e</sup> mice after 12 weeks WD**

<b>12 weeks WD Blood</b>	<b><i>Apoe</i><sup>-/-</sup></b>	<b><i>Apoe</i><sup>-/-</sup> <i>ChemR23</i><sup>o/e</sup></b>	<b><i>P</i>-value</b>
Cholesterol (mg/dL)	1827.0 ± 141.9	1722.0 ± 142.1	0.604
Triglycerides (mg/dL)	126.5 ± 12.1	167.9 ± 19.1	0.079
Body weight (g)	28.5 ± 0.8	27.8 ± 1.0	0.572
Leukocytes (cells x 10 <sup>6</sup> /ml blood)	2.84 ± 0.3	2.74 ± 0.3	0.830
Neutrophils (% of leukocytes)	17.6 ± 0.9	17.78 ± 1.2	0.698
Monocytes (% of leukocytes)	5.8 ± 0.5	8.2 ± 0.4	0.001
T cells (% of leukocytes)	13.3 ± 1.6	11.4 ± 1.0	0.323

**Table SII.** Listed are plasma cholesterol and triglycerides levels, body weight, total leukocytes counts and the percentage of neutrophils, monocytes and T cells in peripheral blood of *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>o/e</sup> mice after 12 weeks of WD (n= 20-23); Data represent mean±SEM, as analyzed by Student's t-test with Welch correction or Mann-Whitney test, as appropriate.

**Supplemental Table III. Blood parameters of *Apoe*<sup>-/-</sup> vs. *Apoe*<sup>-/-</sup>*ChemR23*<sup>wt/e</sup> mice after 4 weeks WD**

<u>4 weeks WD</u> <u>Blood</u>	<i>Apoe</i> <sup>-/-</sup>	<i>Apoe</i> <sup>-/-</sup> <i>ChemR23</i> <sup>wt/egfp</sup>	<i>P</i> -value
Cholesterol (mg/dL)	761.4 ± 22.8	689.4 ± 41.9	0.160
Triglycerides (mg/dL)	71.7 ± 4.7	71.45 ± 3.6	0.957
Body weight (g)	22.1 ± 06	24.7 ± 1.2	0.100
Leukocytes (cell/ml blood)	2.46*10 <sup>6</sup> ± 0.3 10 <sup>6</sup>	2.26*10 <sup>6</sup> ± 0.2*10 <sup>6</sup>	0.662
Neutrophils (% of leukocytes)	21.8 ± 1.2	25.5 ± 1.8	0.109
Monocytes (% of leukocytes)	12.6 ± 1.4	13.8 ± 1.0	0.499
T cells (% of leukocytes)	22.7 ± 1.3	21.2 ± 0.8	0.406

**Table SIII.** Listed are plasma cholesterol and triglycerides levels, body weight, total leukocytes counts and the percentage of neutrophils, monocytes and T cells in peripheral blood of *Apoe*<sup>-/-</sup> *ChemR23*<sup>wt/e</sup> or *Apoe*<sup>-/-</sup> mice after 4 weeks of WD (n=8-9); Data represent mean±SEM.

**Supplemental Table IV. Blood parameters of *Apoe*<sup>-/-</sup> recipients after reconstitution with *Apoe*<sup>-/-</sup> vs. *Apoe*<sup>-/-</sup> *ChemR23*<sup>eg/eg</sup> bone marrow and 6 weeks WD**

<b>BMT 6 weeks WD Blood</b>	<b><i>Apoe</i><sup>-/-</sup> &gt; <i>Apoe</i><sup>-/-</sup></b>	<b><i>Apoe</i><sup>-/-</sup> <i>ChemR23</i><sup>egfp/egfp</sup> &gt; <i>Apoe</i><sup>-/-</sup></b>	<b>P-value</b>
Cholesterol (mg/dL)	1761.0 ± 107.0	1783.0 ± 76.6	0.871
Triglycerides (mg/dL)	247.3 ± 27.8	242.9 ± 32.9	0.671
Body weight (g)	23.9 ± 0.4	24.0 ± 0.3	0.832
Leukocytes (cells x 10 <sup>6</sup> /ml blood)	3.57 ± 0.4	2.45 ± 0.4	0.019
Neutrophils (% of leukocytes)	17.8 ± 0.7	19.1 ± 0.7	0.206
Monocytes (% of leukocytes)	8.8 ± 0.4	11.9 ± 0.8	<0.0001
T cells (% of leukocytes)	10.8 ± 0.5	12.3 ± 1.0	0.191

**Table SIV.** Listed are plasma cholesterol and triglycerides levels, body weight, total leukocytes counts and the percentage of neutrophils, monocytes and T cells in peripheral blood of *Apoe*<sup>-/-</sup> mice receiving either *Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> *ChemR23*<sup>eg/eg</sup> bone marrow and 6 weeks of WD (n = 23-28); Data represent mean±SEM, as analyzed by Student's t-test with Welch correction or Mann-Whitney test, as appropriate.