Brief Report

Hematopoietic ChemR23 (Chemerin Receptor 23) Fuels Atherosclerosis by Sustaining an M1 Macrophage-Phenotype and Guidance of Plasmacytoid Dendritic Cells to Murine Lesions

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Objective—Expression of the chemokine-like receptor ChemR23 (chemerin receptor 23) has been specifically attributed to plasmacytoid dendritic cells (pDCs) and macrophages and ChemR23 has been suggested to mediate an inflammatory immune response in these cells. Because chemokine receptors are important in perpetuating chronic inflammation, we aimed to establish the role of ChemR23-deficiency on macrophages and pDCs in atherosclerosis.

Approach and Results—ChemR23-knockout/knockin mice expressing eGFP (enhanced green fluorescent protein) were generated and after crossing with apolipoprotein E-deficient (*Apoe*$^{-/-}$ *ChemR23*$^{e/e}$) animals were fed a western-type diet for 4 and 12 weeks. *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice displayed reduced lesion formation and reduced leukocyte adhesion to the vessel wall after 4 weeks, as well as diminished plaque growth, a decreased number of lesional macrophages with an increased proportion of M2 cells and a less inflammatory lesion composition after 12 weeks of western-type diet feeding. Hematopoietic ChemR23-deficiency similarly reduced atherosclerosis. Additional experiments revealed that ChemR23-deficiency induces an alternatively activated macrophage phenotype, an increased cholesterol efflux and a systemic reduction in pDC frequencies. Consequently, expression of the pDC marker SiglecH in atherosclerotic plaques of *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice was declined. ChemR23-knockout pDCs also exhibited a reduced migratory capacity and decreased CCR (CC-type chemokine receptor)7 expression. Finally, adoptive transfer of sorted wild-type and knockout pDCs into *Apoe*$^{-/-}$ recipient mice revealed reduced accumulation of ChemR23-deficient pDCs in atherosclerotic lesions.

Conclusions—Hematopoietic ChemR23-deficiency increases the proportion of alternatively activated M2 macrophages in atherosclerotic lesions and attenuates pDC homing to lymphatic organs and recruitment to atherosclerotic lesions, which synergistically restricts atherosclerotic plaque formation and progression. (***Arterioscler Thromb Vasc Biol*. 2019;39:00-00. DOI: 10.1161/ATVBAHA.119.312386.)**

Key Words: atherosclerosis ■ chemokines ■ dendritic cells ■ macrophages ■ mice

Atherosclerosis is a western lifestyle associated inflammatory disease of the arterial wall and responsible for ischemic cardiovascular diseases and stroke. Over the last years, a large body of evidence has shown that innate and adaptive immunity play a crucial role in atherosclerotic lesion formation and progression. During early steps of atherogenesis, the subendothelial retention of modified lipids promotes the recruitment of leukocytes into the activated intima. Monocytes, for example, differentiate into macrophages and eventually into lipid-laden foam cells which promote plaque development and vulnerability, while antigen presenting cells such as dendritic cells (DCs) induce an adaptive immune response.[1] In

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Nonstandard Abbreviations and Acronyms

Apoe	apolipoprotein E
CCL	CC-type chemokine ligand
CCR	CC-type chemokine receptor
ChemR23	Chemerin Receptor 23
CXCL	CXC-type chemokine ligand
CXCR	CXC-type chemokine receptor
DC	Dendritic cell
DiI-LDL/oxLDL	1′-dioetadeeyl-3,3,3′,3′-tetramethylindocarboeyanine perchlorate/oxidized low-density lipoprotein
EC	endothelial cell
eGFP	enhanced green fluorescent protein
EPA	eicosapentaenoic acid
HDL	high-density lipoprotein
ICAM1	intercellular Adhesion Molecule 1
IFN	interferon
pDC	plasmacytoid dendritic cell
RvE1	resolvin E1
WD	western-type diet

this context also chemokines and their receptors have gained attention and have been implicated in the (patho)physiology of atherosclerosis.[2,3]

The chemokine-like receptor ChemR23 (chemerin receptor 23) is a G protein-coupled receptor for the chemoattractant adipokine chemerin and the proresolving molecule, resolvin E1 (RvE1).[4,5] ChemR23 is expressed by (immature) DCs, among which mainly by unstimulated plasmacytoid dendritic cells (pDCs), and by (inflammatory) macrophages.[6,7] The ChemR23 ligand chemerin has been identified as a chemotactic factor for pDCs[8,9] and is secreted as a proprotein that then undergoes several proteolytic cleavages. Different chemerin variants with proinflammatory or anti-inflammatory action can be produced, depending on the class of proteases predominating in the microenvironment.[10] Concomitantly, engagement of ChemR23 has been reported to play a pathophysiological role in more chronic inflammatory diseases, such as lupus, psoriasis, or multiple sclerosis, while it seems to exert beneficial effects in lung inflammation and viral infections.[4] In terms of cardiovascular diseases it was, for example, shown that local chemerin expression within the vascular wall of human plaque specimens[11] and epicardial adipose tissue[12] correlates with the severity of coronary atherosclerosis. Furthermore, chemerin has been implicated in inducing apoptosis of cardiomyocytes,[13] modifying vasoconstriction,[14] and increasing blood pressure,[15] most likely via ChemR23. However, an isoform of chemerin (chemerin15) has been reported to induce alternatively activated M2 macrophage polarization, thereby protecting against myocardial ischemia-reperfusion injury in mice.[16] Another recent study revealed exacerbated atherosclerotic lesion formation in ChemR23-deficient animals, attributing this increase to the lost interaction of ChemR23 with its alternative proresolving ligand RvE1. Supplementation experiments with eicosapentaenoic acid (EPA) as a precursor for the proresolving RvE1 indeed reduced plaque formation in Apoe-deficient mice. However, EPA supplementation was not investigated in ChemR23-deficient mice, leaving the role of ChemR23 in the observed EPA-induced effects on atherosclerosis unanswered.[17]

Although several aspects of the ChemR23/chemerin axis with respect to cardiovascular diseases have been investigated, the outcome of ChemR23 expression on pDCs and macrophages, which are the 2 main leukocyte subsets expressing the receptor, has not been addressed in the context of atherosclerosis. Therefore, we generated a ChemR23 reporter mouse model and investigated the role of ChemR23 in atherogenesis and progression in more detail.

Materials and Methods

In adherence to the American Heart Association 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$^{e/e}$ 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. *ChemR23$^{e/e}$* mice were crossed with *Apoe$^{-/-}$* mice to generate *ChemR23$^{e/e}$ Apoe$^{-/-}$* mice (all on C57BL/6 background). For atherosclerosis studies, mice were fed a western-type diet (WD) containing 21% fat and 0.15% to 0.2% cholesterol, starting at 8 to 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.[18] All animal experiments were approved by the local ethical committee (Regierung von Oberbayern, Sachgebiet 54, Germany).

Histology and Immunofluorescence

Atherosclerotic lesion size was assessed by analyzing cryosections of the aortic root by staining for lipid depositions with Oil-Red-O. 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-staining.

Intravital Microscopy

Leukocyte adhesion to the carotid artery was analyzed using intravital microscopy, as described.[18]

Laboratory Blood Parameters and Flow Cytometry

EDTA-buffered blood was obtained from *Apoe$^{-/-}$* control or *ChemR23$^{e/e}$ Apoe$^{-/-}$* mice and selected blood parameters were determined using an automated blood cell counter. Bone marrow cells were harvested by flushing femurs, spleen, and lymph nodes were mechanically crushed and filtered to obtain single cell suspensions. All cell suspensions were analyzed using a FACS Canto II.

Functional Analysis of pDCs and Aortic Macrophages

pDCs are isolated from bone marrow, and macrophages are isolated from the aortic tree or aorta using FACS sorting. Cells were further processed for polymerase chain reaction, cell culture, or flow cytometric analysis.

Adoptive Transfer

Sorted pDCs are stained using cell tracker proliferation dye eF670. Recipient *Apoe$^{-/-}$* mice are fed a WD for 10 weeks. Labeled pDCs are injected intravenous (≈5×10^5 cells/recipient) single cell suspensions of excised aortas are analyzed 24 hours after injection by flow cytometry.

Bone Marrow-Derived Macrophages and Cholesterol Efflux/Uptake

Bone marrow was isolated from femurs and tibiae of C57BL/6 control or *ChemR23$^{e/e}$* mice. Cells were cultured for 8 to 9 days to differentiate into bone marrow-derived macrophages. ABCA1/ABCG1 surface expression was analyzed using flow cytometry. For the cholesterol efflux assay, cultured bone marrow-derived macrophages were replated at 0.5×10^6 cells per well in a 24-well plate and allowed to adhere overnight. Next day the culture medium was replaced by medium containing tritium (3H)-cholesterol (1 µCi/well); and oxLDL (oxidized low-density lipoprotein; 50 µg/mL) for 24 hours. After loading the cells were equilibrated for 2 hours in RPMI medium containing 0.2% BSA. Subsequently, cells were incubated for 6 hours with RPMI medium containing 0.2% BSA and ApoA1 (15 µg/mL) or HDL (high-density lipoprotein; 50 µg/mL) or received no further treatment. Medium was removed, collected, and the cells were lysed at 37°C with 0.3 mol/L sodium hydroxide solution for 15 minutes. Cell lysate was collected and both, the lysate and the supernatant were transferred to scintillation medium and radioactivity was 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.

Statistics

All data are expressed as mean±SEM. Statistics were performed using of GraphPadPrism7. Normality was tested and unpaired Student *t* test with Welch correction, Mann-Whitney test, 1-way ANOVA, or Kruskal-Wallis with Dunnett or Dunn multiple comparisons or 2-way ANOVA with Sidak multiple comparisons test were used, as appropriate. *P* values <0.05 were considered as being statistically significant.

A more detailed version of Materials and Methods is available in the online-only Data Supplement.

Results

ChemR23 Is Expressed on pDCs and Macrophages

To study ChemR23 expression on different cell types and to investigate the deletion of the ChemR23 receptor in an atherosclerotic context, we generated ChemR23-knockout/knockin reporter mice expressing the eGFP (enhanced green fluorescent protein) instead of ChemR23 (Figure I in the online-only Data Supplement). Subsequently, we bred these mice to apolipoprotein E-deficient (*Apoe$^{-/-}$*) animals to generate ChemR23/Apoe double-deficient animals (*Apoe$^{-/-}$ ChemR23$^{e/e}$*) for our atherosclerosis studies. Next, we evaluated ChemR23 (eGFP) expression on different hematopoietic cell types and confirmed its expression on pDCs[8] and macrophages[19] in *ChemR23$^{e/e}$* and *Apoe$^{-/-}$ ChemR23$^{e/e}$* mice (Figure IIA in the online-only Data Supplement),[7] while monocytes and neutrophils did not express the receptor (Figure IIB in the online-only Data Supplement).

ChemR23-Deficiency Reduces Atherosclerotic Lesion Formation After 4 and 12 Weeks WD Feeding Accompanied With Less Inflammatory Lesion Composition

To assess if and how ChemR23 impacts atherosclerotic lesion development, we subjected *Apoe$^{-/-}$* and *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals to WD feeding for 4 and 12 weeks. We observed a significant reduction in lesion size in the aortic root and the aortic arch after 4 weeks WD (Figure 1A through 1C) along with a decline in lesional macrophage numbers (Figure 1D) and a decreased expression of the ICAM1 (intercellular adhesion molecule 1) on intimal endothelial cells (ECs; (Figure IIIA in the online-only Data Supplement). In line with this, adhesion of CD11b-positive myeloid cells including Ly6C-positive monocytes (Figure 1E and 1F) and Ly6G-positive neutrophils (Figure IIIB in the online-only Data Supplement) to inflamed arterial endothelium was significantly diminished in *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals as revealed by intravital microscopy[20] of the carotid artery. Paralleling these findings after 4 weeks WD, atherosclerotic plaque formation in aortic roots and arches was also reduced in ChemR23-deficient mice after 12 weeks WD (Figure 1G through 1I). This was associated with a reduction in the number of lesional macrophages (Figure 1J) despite an increased presence of CD206+ macrophages in *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals (Figure IVA in the online-only Data Supplement), pointing at a greater abundance of M2 macrophages but reduced presence of M1 macrophages in lesions of ChemR23-deficient animals. Further, necrotic core size in lesions was significantly diminished on ChemR23-knockout (Figure 1K) while the number of TUNEL+ cells was not affected (Figure IVB in the online-only Data Supplement). Collagen content (Figure 1L), the number of smooth muscle cells (Figure IVC in the online-only Data Supplement) and the thickness of the fibrous cap (Figure IVD in the online-only Data Supplement) were significantly enhanced in lesions of *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals compared with control mice after 12 weeks WD. Combined, these results underline less inflammatory lesion composition and a reduced inflammatory immune response in ChemR23-deficient animals in both early and advanced atherosclerosis. While total cholesterol levels, total leukocyte counts, and body weight did not differ on ChemR23-deficiency, neither after 4 weeks WD (Table I in the online-only Data Supplement) nor after 12 weeks WD (Table II in the online-only Data Supplement), pDC frequencies were significantly reduced in blood, spleen, bone marrow, and lymph nodes of ChemR23-deficient mice in both atherosclerosis studies (Figure 1M and 1N; Figure IVE in the online-only Data Supplement). In contrast, monocyte numbers were not altered after 4 weeks WD, but significantly enhanced in blood while reduced in bone marrow after 12 weeks WD (Figure 1O), suggesting increased mobilization of monocytes from the bone marrow to the blood after 12 weeks WD. Nevertheless, chemerin levels were mildly increased after 4 and 12 weeks of WD on ChemR23-deficiency (Figures IIIC and IVF in the online-only Data Supplement). Heterozygous deficiency of ChemR23 did not change lesion size or any other parameters examined (Figure IIID through IIIF and Table III in the online-only Data Supplement).

In summary, despite mild monocytosis in *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals on WD, atherosclerotic lesion formation is reduced, associated with a reduction in lesional macrophages and circulating pDC levels.

Hematopoietic ChemR23 Fuels Lesion Development

Recent studies have suggested that also vascular ECs and smooth muscle cells express ChemR23.[21,22] Therefore, to examine the role of specifically hematopoietic ChemR23-deficiency in the

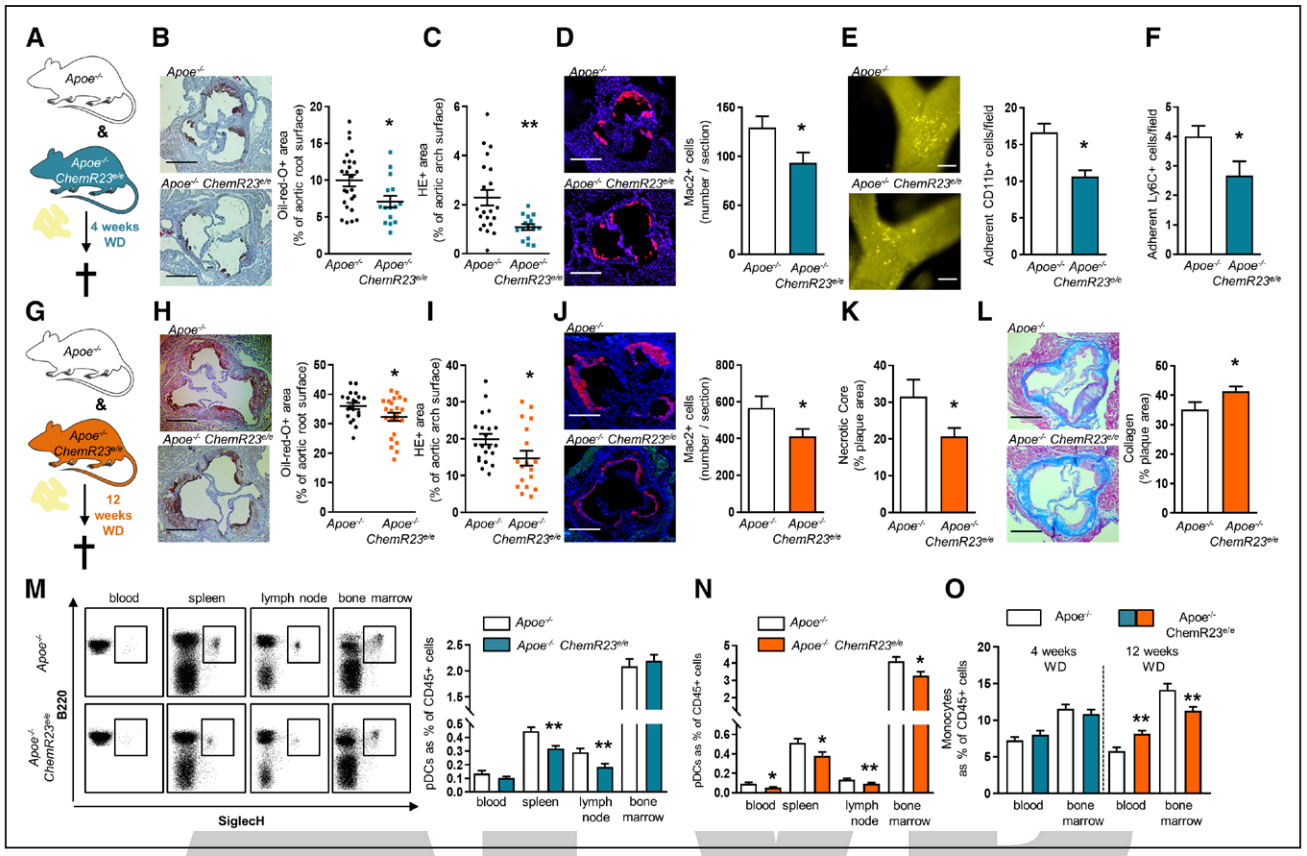

Figure 1. ChemR23 (chemerin receptor 23)-deficiency reduces atherosclerotic plaque size and diminishes lesional inflammation after 4 and 12 wk western-type diet (WD) in *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice. **A**, Experimental scheme of 4 wk WD; (**B**) Representative pictures and quantification of lesion area measured after Oil-Red-O staining for lipid deposits in the aortic root of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice after 4 wk WD. Scale bar =500 µm; (**C**) Atherosclerotic lesion size in aortic arches, quantified using H&E staining of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice after 4 wk WD; (**D**) Representative pictures and quantification of macrophage content in aortic root lesions of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, as quantified after Mac2 staining after 4 wk WD (n=15–20). Scale bar =500 µm; (**E** and **F**) Representative images and quantification of intravital microscopy of leukocyte adhesion to TNF-α–stimulated carotid arteries of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice after 4 wk WD (n=6 per group). Leukocyte labeling was performed for (**E**) CD11b (all myeloid cells), or (**F**) Ly6C (monocytes). Scale bar =100 µm; (**G**) Experimental scheme of 12 wk WD; (**H**) Representative pictures and quantification of lesion area in the aortic roots of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, measured after Oil-Red-O staining for lipid deposits after 12 wk WD. Scale bar =500 µm; (**I**) Atherosclerotic lesion size measured after H&E staining in aortic arches of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice after 12 wk WD; (**J**) Representative pictures and quantification of macrophage content in aortic root lesions of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, quantified after Mac2 staining after 12 wk WD (n=18–21). Scale bar =500 µm; (**K**) Necrotic core area in aortic root lesions of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, as quantified by anucleated area measurement (n=14–17); (**L**) Collagen content in aortic root lesions of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, measured using Masson-Trichrome staining after 12 wk WD (n=14–15); (**M** and **N**) Representative dot plots and quantification of plasmacytoid dendritic cells (pDCs) by flow cytometry in different organs of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice, pregating CD45, after 4 wk (**M**; n=14–20) or 12 wk (**N**; n=18–21) WD; Data represent mean±SEM. **P*<0.05; ***P*<0.01, as analyzed by Student *t* test with Welch correction or Mann-Whitney test, as appropriate.

observed differences in plaque formation, we performed bone marrow transplantations of *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ bone marrow into *Apoe*$^{-/-}$ mice with subsequent WD feeding for 6 weeks. Again we observed a significant reduction in atherosclerotic lesion formation (Figure VA through VC in the online-only Data Supplement), diminished lesional macrophage content (Figure VD in the online-only Data Supplement) and a decline in pDC frequencies in blood, lymph node, and bone marrow (Figure VE in the online-only Data Supplement). Concordant with our findings on total ChemR23-deficiency after 4 and 12 weeks WD, the relative number of monocytes was significantly increased in animals receiving ChemR23-deficient bone marrow (Table IV in the online-only Data Supplement). Thus, the observation that hematopoietic ChemR23-deficiency mimics total ChemR23-knockout underlines the importance of hematopoietic ChemR23 expression in the observed proatherogenic immune response. Supportive of this notion, we could detect in our immunofluorescent analyses of root lesions of *ChemR23*$^{e/e}$ reporter mice an eGFP signal in lesional macrophages and pDCs (Figure VIA and VIB in the online-only Data Supplement). Hence, we conclude that hematopoietic ChemR23 expression fuels lesion development.

ChemR23 Controls Macrophage Polarization in Atherosclerotic Lesions

To unravel mechanisms responsible for reduced lesion formation in ChemR23-deficient animals, we evaluated the macrophage phenotype in more detail, because ChemR23 expression has been suggested to be specific for M1 macrophages at least in vitro[6] and we could detect more CD206+ macrophages in ChemR23-deficient lesions despite a general reduction in lesional macrophages. To this end, we sorted CD11b+F4/80+ macrophages from aortic cell suspensions (arch and thoracal aorta) of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ animals after 12 weeks WD and found the number of aortic macrophages to be significantly reduced in *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ animals

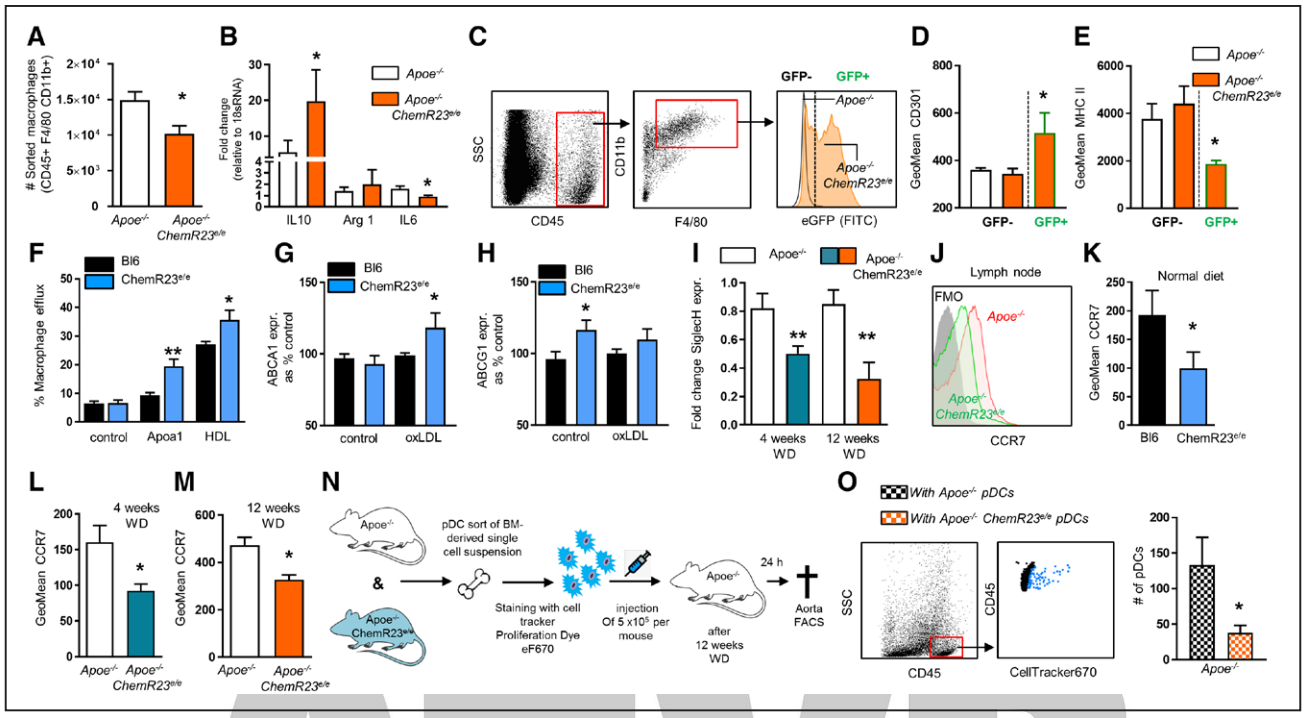

Figure 2. ChemR23 (chemerin receptor 23)-deficiency sustains macrophage M2 phenotype and reduces plasmacytoid dendritic cell (pDC) recruitment to atherosclerotic lesions. **A**, Macrophages were FACS sorted from the aortic tree of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice fed a western-type diet (WD) for 12 wk (n=11–17 animals per group); (**B**) Real-time mRNA expression analysis of sorted macrophages for the indicated genes; (**C**) Gating strategy for aortic macrophages analyzed by flow cytometry in aortic cell suspensions from *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ fed a WD for 12 wk; flow cytometric evaluation of CD301 (**D**) and MHCII (**E**) expression quantified as geometric mean (GeoMean) on eGFP (enhanced green fluorescent protein)-negative (GFP–) and eGFP-positive (GFP+) aortic macrophages from *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ fed a WD for 12 wk (n=5 per group); (**F**) Cholesterol efflux to Apoa1 (apolipoprotein A1) in bone marrow-derived macrophages from C57BL/6 (Bl6) and ChemR23-deficient animals (n=6–9 independent experiments); flow cytometric analysis of geometric mean fluorescence intensity of ABCA1 (**G**) and ABCG1 (**H**) expression on bone marrow-derived macrophages from Bl6 and ChemR23-deficient animals (n=7 independent experiments), where indicated after stimulation with oxLDL (oxidized low-density lipoprotein; 25 μg/mL) for 4 h; (**I**) Evaluation of SiglecH mRNA expression by real-time polymerase chain reaction in aortic root sections of *Apoe*$^{-/-}$ or *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ fed a WD for 4 or 12 wk (n=8–10 per group); (**J**) Representative histogram of CCR (CC-type chemokine receptor)7 expression on lymphatic pDCs in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ animals; Quantification of CCR7 as geometric mean fluorescence intensity (GeoMean) on pDCs from lymph nodes of Bl6 and ChemR23-deficient animals (**K**, n=8 per group), of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice after 4 wk WD (**L**, n=16) and 12 wk WD (**M**, n=7–12); (**N**) Experimental scheme for the adoptive transfer experiment with sorted pDCs; (**O**) Representative gating strategy for adoptively transferred pDCs (labeled with cell tracker Proliferation dye eF670) (**left**, depicted are large dots) and quantification of adoptively transferred *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ pDCs in aortic cell suspensions from *Apoe*$^{-/-}$ mice receiving a WD for 12 wk (n=7–8 per group); Data represent mean±SEM. **P*<0.05; ***P*<0.01, as analyzed by Student *t* test with Welch correction, Mann-Whitney, Kruskal-Wallis with Dunn multiple comparisons test or 2-way ANOVA with Sidak multiple comparisons test, as appropriate.

(Figure 2A), paralleling our findings in aortic root lesions (Figure 1D and 1J). Gene expression analysis of sorted macrophages furthermore revealed ChemR23-deficiency to cause a significant upregulation of *Il10* mRNA expression as a marker of the alternative M2 profile, while the expression of *Il6*, indicative of a proinflammatory M1 profile, was significantly reduced (Figure 2B). A flow cytometric evaluation of the macrophage phenotype in the aortic tree on 12 weeks WD showed again, this time with removal of the adventitia, a diminished total number of macrophages in *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice (data not shown), and when distinguishing between eGFP– and eGFP+ macrophages using flow cytometry, around 50% of aortic macrophages were found to be GFP+ in *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice (Figure VIC in the online-only Data Supplement). Surface expression of CD301 (M2 marker) and MHCII (M1 marker) was significantly upregulated, respectively, downregulated within the GFP+ macrophage compartment compared with GFP– macrophages or the *Apoe*$^{-/-}$ macrophages in general (Figure 2C through 2E). The pronounced M2 phenotype in ChemR23-deficient but GFP+ macrophages points towards an important role of the chemerin/ChemR23 axis in controlling macrophage polarization and induction of an M1 phenotype in (chronic) inflammation, underlining findings from Lin et al[23] showing that chemerin blocks M2 polarization in colitis. To verify these findings independent of Apoe- and ChemR23-deficiency, we injected PBS, TNF-α, and chemerin into the peritoneal cavity of Bl6 animals and analyzed macrophage numbers as well as CD301 and MHCII surface expression after 4 hours. Here, we revealed an increased number of (recruited) macrophages in the peritoneal cavity of TNF-α and chemerin treated animals and, in contrast to our findings in ChemR23-deficient mice, a significant decrease in the expression of CD301, while MHCII expression was enhanced (Figure VIIA through VIID in the online-only Data Supplement). In addition, chemerin treatment significantly augmented apoptosis (AnnexinV+) in macrophages from wild type but not from ChemR23-deficient animals (Figure VIIE in the online-only Data Supplement), which may explain the reduced necrotic core size in lesions of *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ animals (Figure 1K).

Further, with M1 macrophage polarization reported to reduce cholesterol efflux capacity,[24] we examined the effect

of ChemR23-deficiency on cholesterol efflux. As shown in Figure 2F, efflux of ^{3}H-cholesterol to Apoa1 (apolipoprotein A1) or HDL was significantly enhanced in *ChemR23$^{e/e}$* macrophages compared with control cells, in accordance with an increased M2 polarization on ChemR23-deficiency (Figure 2D and 2E). While surface expression of the efflux transporter ABCA1 only increased on ChemR23-deficiency after oxLDL treatment, ABCG1 already increased under baseline conditions in ChemR23-deficient macrophages (Figure 2G and 2H). In contrast to the observed effects of ChemR23-deficiency on cholesterol efflux, uptake of Dil-labeled LDL (low-density lipoprotein) or Dil-labeled oxLDL was not altered between control and ChemR23-deficient macrophages (Figure VIIIA in the online-only Data Supplement). Also, the efferocytotic capacity of macrophages was not affected by ChemR23-deficiency (Figure VIIIB and VIIIC in the online-only Data Supplement).

Taken together, the absence of ChemR23 favors the M2 macrophage phenotype and increases the proportion of alternatively activated macrophages in atherosclerotic lesions, associated with a reduced plaque formation.

ChemR23-Deficiency Decreases CCR7 Expression on pDCs and Limits Their Recruitment to Atherosclerotic Lesions

The involvement of chemokine receptors in the migration of pDCs has been controversially discussed.[25] However, it can be agreed on that ex vivo isolated pDCs (before activation, eg, through TLR7 or TLR9 engagement) express the chemokine receptors CCR (CC-type chemokine receptor)2, CCR5, CCR7, CXCR (CXC-type chemokine receptor)3, CXCR4, and ChemR23, while they only migrate in response to CXCL (CXC-type chemokine ligand)12 (receptor CXCR4) and chemerin (receptor ChemR23).[26,27] Stimulated pDCs mainly respond to the CCR7 ligands CCL (CC-type chemokine ligand)19 and CCL21, and CCR7 does also essentially contribute to the homing of pDCs to lymph nodes in steady-state and inflammation.[28] However, also ChemR23 has been reported to direct pDC migration to lymphatic organs and, even more important, specifically to sites of inflammation.[9,27] As pDC frequencies were reduced in lymphatic organs on ChemR23-deficiency in all our atherosclerosis models (Figure 1M and 1N; Figure VE in the online-only Data Supplement) and sialic acid binding Ig-like lectin (SiglecH) mRNA expression as pDC marker was significantly reduced in aortic root lesions after 4 and 12 weeks WD (Figure 2I), we followed up on the migratory capacity of ChemR23-competent and -deficient pDCs. Transmigration assays with FACS-sorted pDCs from Bl6, *ChemR23$^{e/e}$*, *Apoe$^{-/-}$*, and *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals and revealed that ChemR23-deficiency abolished chemerin-induced migration of pDCs without affecting CXCL12-triggered pDC migration (Figure IXA and IXB in the online-only Data Supplement). Similarly, migration of CpG-treated *Apoe$^{-/-}$ ChemR23$^{e/e}$* pDCs in response to CCL19 (ligand of CCR7) was observed only in ChemR23-competent cells and not on ChemR23-deficiency (Figure IXC in the online-only Data Supplement).

Next, we examined CXCR4 and CCR7 expression on lymphatic ChemR23-deficient pDCs in comparison to control cells and observed a significant decrease in CCR7 expression on *ChemR23$^{e/e}$* and *Apoe$^{-/-}$ ChemR23$^{e/e}$* on pDCs in steady-state (Figure 2J and 2K) and under atherosclerotic conditions (Figure 2L and 2M). Expression of CXCR4 did not differ between pDCs from control and knockout animals (data not shown). We further evaluated if ChemR23-deficiency does also impact on other aspects of pDC phenotype such as type I IFN (interferon) production or expression of MHCII and CD86. As depicted in Figure XA and XB in the online-only Data Supplement, CpG stimulation of sorted pDCs for 12 hours still induced a strong IFN-α response in ChemR23-deficient cells and comparable upregulation of MHCII and CD86 expression in wild-type and knockout pDCs. Also, chemerin treatment did not induce type I IFN, MHCII, or CD86 expression in pDCs (data not shown). In addition, neither CpG nor chemerin treatment enhanced pDC apoptosis in control nor ChemR23-deficient pDCs (Figure XC in the online-only Data Supplement). To validate the extent to which migration of ChemR23-deficient pDCs to atherosclerotic lesions is affected in vivo, we sorted pDCs from *Apoe$^{-/-}$* and *Apoe$^{-/-}$ ChemR23$^{e/e}$* animals (Figure XI in the online-only Data Supplement), labeled them and injected these cells into *Apoe$^{-/-}$* recipients with established lesions (Figure 2N). After 24 hours, the amount of labeled cells in aortic cell suspensions was quantified by flow cytometry, revealing a significant reduction in the number of lesional pDCs in *Apoe$^{-/-}$* recipients that received ChemR23-deficient pDCs (Figure 2O).

In summary, ChemR23-deficiency reduces CCR7 expression on pDCs, hinders their migration triggered by chemerin and CCL19 as well as their recruitment into atherosclerotic lesions and homing to lymphatic organs. Consequently, pDC-mediated proinflammatory immune responses are restricted.[29] In contrast to previous findings, relating exacerbated lesion formation in ChemR23-deficient mice to the interaction of ChemR23 with the alternative proresolving ligand RvE1, the reduction of atherosclerosis observed in our study is likely because of the effects of its canonical ligand chemerin.

Discussion

This study provides evidence that reduced atherosclerosis on ChemR23-deficiency in *Apoe$^{-/-}$* mice is accompanied by reduced leukocyte adhesion to inflamed vessels and a less inflammatory plaque phenotype. In addition, we observed an increased abundance of alternatively activated macrophages in atherosclerotic lesions and a systemic reduction in pDC frequencies, as well as a reduced migratory capacity of ChemR23-deficient pDCs to sites of inflammation. We, therefore, conclude that the absence of ChemR23-mediated signaling favors M2 polarization in atherosclerotic lesions and attenuates pDC homing to lymphatic organs and recruitment to sites of inflammation, which synergistically restricts atherosclerotic plaque formation and progression.

Most studies evaluating the impact of the chemerin/ChemR23 axis on cardiovascular disease have focused on the chemotactic adipokine chemerin.[11–15,30] As already outlined in the introduction, different chemerin variants are generated

depending on the protease type cleaving the proform. These variants vary in their biological activity and include proinflammatory and anti-inflammatory isoforms, which may outweigh each other depending on the stage of the inflammatory process and the predominant metabolizing enzymes present.[10] For example, chemerin15 has been associated with beneficial effects in mouse myocardial reperfusion injury by inducing alternative macrophage polarization.[16] Others report suppression of M2 polarization by chemerin,[23] and in vitro studies suggest that ChemR23 expression is a marker of inflammatory macrophages.[6] Notably, we see enhanced chemerin levels in plasma (Figures IIIC, IVD, and VF in the online-only Data Supplement) and bone marrow lavages (data not shown) in all our ChemR23-deficiency models independent of lesion size, suggesting that (pro)-chemerin accumulates because its main receptor is lacking and a negative feedback loop preventing its further generation is missing. However, despite increased levels of chemerin in our ChemR23-deficient mice, pDCs are impaired in numbers as well as in their migratory response to chemerin and homing in lymphatic organs, underlining the important role of the ChemR23/chemerin axis in pDC homing and recruitment.[7–9,27,31] In addition, macrophages in our study reveal an alternative phenotype profile, an increased cholesterol efflux capacity as well as a defective chemerin-induced transmigration on ChemR23-deficiency. This again supports the idea that the ChemR23/chemerin axis plays a central role in shaping macrophage-mediated immune responses,[6,19] and, taking into account previous findings on chemerin15 inducing alternative macrophage polarization,[16] the ChemR23-mediated response in macrophages may be proinflammatory or anti-inflammatory depending on the main protease processing prochemerin. However, in chronic inflammation such as atherosclerosis, inflammation resolution is disturbed because of a reduced or imbalanced presence of proresolving mediators.[32,33] The latter may also apply to the relative abundance of proinflammatory versus anti-inflammatory chemerin variants because of an altered availability of prochemerin-processing proteases under chronic inflammatory conditions. It would be helpful to quantify the individual variants in inflammatory settings; however, this is difficult to achieve because of the small differences in amino acid sequence and structure.

To our knowledge only one other, recent study has addressed the role of ChemR23-deficiency in atherosclerosis. First, Laguna-Fernandez et al[17] show that supplementation of the RvE1-precursor EPA significantly attenuated atherosclerotic lesion growth induced by WD in *Apoe*$^{-/-}$ mice and altered lipoprotein metabolism with a dramatic (50%) reduction in total cholesterol levels. In a complementary approach, they also knockedout ChemR23 in *Apoe*$^{-/-}$ mice and evaluated atherosclerosis after WD feeding, though in a rather limited number of animals.[17] Distinct from their supplementation studies, no changes in total cholesterol levels were observed on ChemR23-deficiency, in accordance with our study. However, surprisingly and in contrast to our findings, Laguna-Fernandez et al[17] report increased lesion formation and macrophage accumulation within atherosclerotic plaques of aortic arches and roots from ChemR23-deficient animals after 8 and 12 weeks WD. Because the mouse model (*Apoe*$^{-/-}$) and the cholesterol content of the diet (≈0.15%) are comparable between this and our study, it remains elusive why the reported results differ in opposite directions. As one potential explanation, plaques in our study were much more advanced, showing a plaque area of ≈10% and 37% of the aortic root surface after 4 and 12 weeks WD, respectively, compared with ≈6% and 13% of aortic root area after 8 and 12 weeks WD, respectively, in the study of Laguna-Fernandez et al.[17] Of note, aortic root lesions in the latter study were only reduced by ChemR23-knockout after 8 but not 12 weeks of WD, with accompanying root lesion sizes (≈6%) below those investigated in our study. This suggests that the atheroprogressive effect of ChemR23-deficiency observed by Laguna-Fernandez et al[17] may only be observed in early stages of atherosclerosis, whereas in stages of larger lesion areas and thus higher inflammatory settings (as in our study already after 4 weeks WD compared with the study of Laguna-Fernandez et al[17]), a protective effect of ChemR23-deficiency may take the overhand. Differential, stage-dependent effects on atherosclerosis have been observed before.[34] As a potential underlying mechanism, the overall outcome of ChemR23-deficiency may be predominated by differential cellular effects depending on the stage of atherosclerosis. As such, the effects of ChemR23-deficiency on hematopoietic cells may overrule in advanced atherosclerosis (as in our study), whereas effects on vascular cells may predominate in earlier stages of atherosclerosis. Our immunohistochemical analyses of ChemR23 in atherosclerotic root lesions indeed also revealed ChemR23 expression in ECs and smooth muscle cells (data not shown), in line with previous reports.[21,22] As chemerin was shown to act anti-inflammatory on ECs,[22] and ECs play an important atheroprotective role already in early stages of atherosclerosis, the absence of anti-inflammatory chemerin/ChemR23 signaling in ECs may be important mostly in early atherosclerosis and contribute to the observed plaque increase in ChemR23-deficient mice in the early atherosclerosis study of Laguna-Fernandez et al.[17] When atherosclerosis progresses, the proinflammatory effects of chemerin/ChemR23 signaling on macrophages and pDCs may gradually increase in importance and eventually take the overhand, resulting in reduced atherosclerosis on ChemR23-deficiency, as observed in our study of more advanced atherosclerosis. A follow-up study addressing the role of ChemR23 specifically in nonhematopoietic cells is warranted to shed more light on cell type-specific effects of ChemR23 in different stages of atherosclerosis.

Further, in the case of ChemR23, a stage-dependent effect of knockout on atherosclerosis might be triggered by an altered balance of its multiple ligands, either being RvE1 or different chemerin variants, in the course of increasing atherosclerosis and inflammation, as discussed above. In this context, Laguna-Fernandez et al[17] unfortunately do not provide a proof of principle study supplying *Apoe*$^{-/-}$ *ChemR23*$^{e/e}$ mice with the RvE1-precursor EPA to investigate whether the observed protective effects of EPA are mediated by ChemR23 or instead by another receptor of RvE1 (eg, BLT1)[35] or are rather related to the high changes in total cholesterol levels observed in *Apoe*$^{-/-}$ mice supplemented with EPA. In the same line, it is possible that the reducing effect of RvE1 on long-term (>40 hours) oxLDL uptake in macrophages, as observed by Laguna-Fernandez et al,[17] contributes to reduced

atherosclerosis on EPA supplementation, with RvE1 in this context expected to take the overhand as ChemR23 ligand. However, without RvE1 addition and at further progressed atherosclerosis stages, a different ChemR23 ligand balance or the absence of intrinsic difference in oxLDL uptake on ChemR23-deficiency as observed in our study, may differentially impact on oxLDL uptake and thereby on atherosclerotic lesion development.

Taken together, our data reveal a proinflammatory role of ChemR23 in atherogenesis and atheroprogression, attributable to the recruitment of pDCs to atherosclerotic lesions and the induction of a proinflammatory lesional macrophage phenotype. These outcomes are in line with other findings in chronic inflammatory diseases,[4,9,11,12,15,23] pointing at an overall proinflammatory role of the ChemR23/chemerin axis in atherosclerosis, probably because of chronic abundance of proteases mediating the generation of chemotactic proinflammatory chemerin isoforms. Nevertheless, the idea of increasing the proportion of anti-inflammatory ligands available for interaction with ChemR23, either by modifying the (local) protease profile or by supplying RvE1, may hold therapeutic potential in chronic inflammatory settings like atherosclerosis.

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Disclosures

None.

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Highlights

- Hematopoietic ChemR23 (chemerin receptor 23)-deficiency reduces both atherogenesis as well as atherosclerotic plaque progression and induces a less inflammatory plaque composition in advanced lesions.
- ChemR23-deficiency on myeloid cells hampers their adhesion to inflamed vessels, favors alternatively activated M2 macrophages in atherosclerotic lesions and enhances cholesterol efflux.
- ChemR23-deficiency on plasmacytoid dendritic cells reduces CCR (CC-type chemokine receptor)7 expression and hinders their homing to lymphatic organs and recruitment to atherosclerotic lesions.