Neutrophils: emerging role in the immunopathology of atherosclerosis

Hartwig, H.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Distinct function of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes

(* Equal contribution)

Contribution Helene Hartwig: assisted during in vivo experimentation, and contributed to tissue processing and analyses and in vitro experiments.

EMBO Molecular Medicine 2013; 5(3):471-81
CHAPTER 3

Abstract

We used a novel approach of cytostatically induced leucocyte depletion and subsequent reconstitution with leucocytes deprived of classical (inflammatory/Gr1\textsuperscript{hi}) or non-classical (resident/Gr1\textsuperscript{lo}) monocytes to dissect their differential role in atheroprospection under high-fat diet (HFD). Apolipoprotein E-deficient (\textit{Apoe}\textsuperscript{-/-}) mice lacking classical but not non-classical monocytes displayed reduced lesion size and macrophage and apoptotic cell content. Conversely, HFD induced a selective expansion of classical monocytes in blood and bone marrow. Increased CXCL1 levels accompanied by higher expression of its receptor CXCR2 on classical monocytes and inhibition of monocytosis by CXCL1-neutralization indicated a preferential role for the CXCL1/CXCR2 axis in mobilizing classical monocytes during hypercholesterolemia. Studies correlating circulating and lesional classical monocytes in gene-deficient \textit{Apoe}\textsuperscript{-/-} mice, adoptive transfer of gene-deficient cells and pharmacological modulation during intravital microscopy of the carotid artery revealed a crucial function of CCR1 and CCR5 but not CCR2 or CX\textsubscript{3}CR1 in classical monocyte recruitment to atherosclerotic vessels. Collectively, these data establish the impact of classical monocytes on atheroprospection, identify a sequential role of CXCL1 in their mobilization and CCR1/CCR5 in their recruitment.
Introduction

Monocytes and their descendants are the most abundant leucocytes in atherosclerotic lesions (1). Studies correlating systemic monocyte counts with severity of atherosclerosis in humans and mice suggest a role of monocytes in disease progression (2, 5). Depletion strategies have provided evidence for the global significance of monocytes in atheroprogression (4), with more recent work indicating a stage-specific influence, whereby monocytes promote atherosclerosis at early stages but not at later time points (5). With the emergence of at least two functionally different monocyte subsets in humans and mice termed classical (inflammatory, Gr1<sup>hi</sup>) and non-classical (resident, Gr1<sup>lo</sup>) monocytes (6), it remains to be determined, which differential impact they have on atherosclerosis.

Hypercholesterolemia selectively increases circulating classical monocyte counts (7) and induces phenotypic changes favoring emigration into atherosclerotic lesions (8), suggesting a prominent role of classical monocytes in atherosclerosis. Fundamental to the importance of monocytes in atherosclerosis is their accumulation within atherosclerotic lesions (1) a process regulated at various levels, i.e. mobilization from sites of production, recruitment, and survival in the lesion (9). Extravasation of monocytes requires the coordinated interaction of selectins, adhesion molecules, and chemokines (10). With the discovery of monocyte subsets a concept has emerged, wherein the relative expression of adhesion molecules or chemokine receptors governs their recruitment behavior. In this context, it has been suggested that classical monocytes, which express higher levels of CCR2 compared to non-classical monocytes (11), are recruited to the site of inflammation in Ccr2<sup>−/−</sup> mice in lower numbers (12). In addition, chemokines and their receptors fulfill important roles in monocyte mobilization from the bone marrow (BM). Accordingly, as CCR2 is essential in mobilization of classical monocytes from the BM, these mice also exhibit markedly reduced numbers of circulating monocytes (13). Beyond recruitment and mobilization, chemokine receptor axes can crucially affect monocyte life span. For instance, absence of CX<sub>3</sub>CR1 results in reduction of non-classical monocytes blood counts, which are restored by introduction of a Bcl2 transgene, suggesting that the CX<sub>3</sub>C axis provides a survival signal (14).

Thus, the objective of this study was to dissect a differential role of monocyte subsets in early stages of atherosclerosis, to clarify the chemokine-
Materials and Methods

Animals

Male Apoe<sup>−/−</sup> mice and chemokine receptor-deficient male Apoe<sup>−/−</sup> mice have been previously described (14-16). All strains were backcrossed for at least 10 generations to the C57Bl/6 background. Mice received HFD (21% fat, 0.15% cholesterol, Altromin) for indicated time points resulting in similar lipid levels in all strains (data not shown).

In vivo experiments

To assess the role of monocyte subsets in atheroprosession, 6 weeks-old Apoe<sup>−/−</sup> mice were fed a HFD for a total of 8 weeks. After 4 weeks of HFD, stable leucopenia was induced by repeated CPM injection (100 mg/kg BW, 2x/week). To reconstitute leucocytes, age- and sex-matched Apoe<sup>−/−</sup> mice receiving HFD for an equal time period were exsanguinated. Blood was labelled with antibodies to CD45, Gr1, and CD115, and individual monocyte subsets were depleted by FACS sorting. Donor leucocytes were injected i.v. 2x/week using one donor mouse per recipient each 1 day after CPM application.

For adoptive transfer studies, classical monocytes were isolated from BM by FACS sorting using antibodies to CD45, CD115, and Gr1. After labelling the monocytes with CFSE, 106 cells were adoptively transferred by tail vein injection to Apoe<sup>−/−</sup> mice. Twenty-four hours after transfer, aortas and hearts of recipient mice were collected for further analysis. For monocyte mobilization studies, rmCXCL1/KC (Peprotech) was injected i.v. at 40 mg/kg. After 1 h, blood was drawn and mice were sacrificed to harvest bones and spleens. Serum CXCL1 in mice was neutralized by daily administration of 5 mg of anti-CXCL1 antibody (clone 124014) or IgG isotype control (clone 54447, both R&D Systems) for 1 week and for 3 consecutive weeks every other day.
Flow cytometry

Staining of single cell suspensions of blood, BM, spleen, or aorta was conducted using combinations of antibodies specific for CCR1-purified (Imgenex, IMG329), CCR2-purified (Epitomics, E68), CXCR2-purified (R&D Systems, clone 242216), CXCR7 (BioLegend, 8F11-M16), CX3CR1 (R&D Systems, AF5825), CCR5-biotinylated (BD, C34-3448), CD115-PE (eBioscience, AFS98), CD11b-PerCp/PE-Cy7 (BD, M1/70), CD44-PerCp (eBioscience, IM7), CD45-APC-Cy (BD, 30-F11), CD45.1-PE-Cy7 (eBioscience, A20), CD45.2-APC (eBioscience, 104), D6-purified (ThermoScientific), F4/80-APC (eBioscience, BM8), CD62L (eBioscience, MEL-14), Gr1-APC/PerCP (eBioscience/BD, RB6-8C5), anti-ratIgG-FITC (eBioscience, 11-4811-85), anti-rabbit IgG-FITC (Sigma–Aldrich), SAVPE-Cy7 (BD). Before cell staining, red blood cell lysis was performed using appropriate volume of lysis buffer (150mM NH4Cl; 10mM KHCO3; 0.1mM diNaEDTA, pH 7.4). Cells were washed with HBSS and directly analyzed by flow cytometry using a FACSCantoII (BD). Absolute cell numbers were assessed by use of CountBrightTM absolute counting beads (Invitrogen). Data were analysed with FlowJo Software (Tree Star Inc.). To assess expression levels of interest, geometrical mean fluorescence intensity (MFI) after subtracting the fluorescence minus one (FMO) control was calculated.

Histology, immunohistochemistry, and immunofluorescence

The extent of atherosclerosis was assessed in aortic root sections by oil-red-O staining (Sigma–Aldrich), followed by computerized image analysis and quantification (Leica Qwin Imaging software). Collagen content was evaluated after Sirius red staining. To define monocyte/macrophage numbers in atherosclerotic plaque area, frozen sections of aortic roots were washed with PBS for at least 5 min followed by an overnight incubation with a 1:400 dilution of anti Mac-2 antibody at 4°C. After incubation with secondary Cy-3 conjugated antibody for 30 min at room temperature, sections were analyzed. To assess the accumulation of CD45.2 donor cells within aortic root sections of CPM treated CD45.1 mice, slides were treated with target retrieval solution (Dako). After blocking, sections were stained for CD45.1 over 60 min at RT with an anti-CD45.1 primary (Abcam, A20) and an anti-mouse FITC-conjugated secondary (Jackson Immunoresearch) antibody. After a second treatment with blocking solution containing mouse serum and avidin sections were subsequently stained with a biotinylated anti-CD45.2 primary (eBioscience, 104) antibody.
and streptavidin- DyLight549 (Vector Laboratories). All sections were analyzed using a Leica DMLB fluorescence microscope and charge couple device (CCD) camera. Furthermore, TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR red (Roche) to assess the number of apoptotic/necrotic cells within aortic root sections.

**Construction of the CX3CR1 antagonist**

An expression construct for the CX3CR1-antagonist F1-fractalkine (17) was ordered at Genscript (Piscataway) as E.coli codon-optimized cDNA cloned in pET26b (Merck). Recombinant F1-fractalkine containing an N-terminal cleavable His-tag (MHHHHHHHWVDDDDK−ILDN...TRNGC20) was expressed in BL21(DE3)Star cells (Invitrogen) cultured in LB medium using a fermenter (Lambda Instruments) for 4 h at 37°C. Insoluble inclusion bodies were washed and isolated by repeated centrifugation, solubilized in Guanidine-HCl and refolded by rapid dilution in native buffer essentially as described (18). The refolded His-F1-fractalkine was enriched by sequential Ni-NTA and cation exchange chromatography. Finally, the leader peptide was removed by overnight digestion with recombinant enterokinase (Merck) (1U/mg protein) and active F1-fractalkine was separated from uncleaved His-F1-fractalkine after cation exchange chromatography. F1-fractalkine was dialysed in 0.1% TFA and stored lyophilized at -30°C.

**Intravital microscopy**

Intravital microscopy of the carotid artery was performed in Cx3cl1egfp/+ Apoe−/− mice as described (19). J113863 to CCR1 (5 mg/kg), RS504393 to CCR2 (5 mg/kg), DAPTA to CCR5 (1 mg/kg), or the above-designed antagonist F1-fractalkine to CX3CR1 (5 mg/kg) were injected i.p. 1 h prior to recording. A PE conjugated antibody to Gr1 (5 mg) was instilled via a jugular vein catheter 15 min prior to recording. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a beam splitter to enable synchronized dual-channel recording, a Hamamatsu 9100-02 EMCCD camera, and a 10x saline-immersion objective. For image acquisition and analysis Olympus cell software was used.
Lipid detection

Serum levels of cholesterol or triglycerides were assessed by EnzyChrom™ Assay Kits (BioAssay Systems). CXCL1 measurements in human plasma samples from patients with moderate hypercholesterolemia and respective controls (20) were analyzed for CXCL1 by use of a commercially available ELISA kit (Quantikine, R&D systems).

ELISA

Different ready-to-use ELISA systems were employed according to manufacturer instructions. Murine MCP-1 and CXCL1 were determined using Quantikine ELISAs (R&D Systems). Serum levels of MCP-3 were detected by Instant ELISA (eBioscience).

PCR Array

Employing RT² Profiler PCR Array (SABiosciences) expression of genes encoding chemokines and their receptors could be investigated. RNA of cells obtained from FACS was isolated using RNeasy Micro Kit (Qiagen) and quantified by measuring the absorbance at 260nm (A260) in a spectrophotometer. Using the RT² First Strand Kit (SABiosciences) cDNA has been generated and checked for quality and efficiency of reverse transcription by RT²RNA QC PCR Array. In case the quality of cDNA met the demands, RT² Profiler PCR Array for mouse chemokines and receptors was performed.

Statistics

All continuous data are expressed as mean ± SD. Statistical calculations were performed using GraphPad Prism 5 (GraphPad Software Inc.). After calculating for normality by D'Agostino Pearson omnibus test either unpaired Student’s t-test, One-way ANOVA with Newman–Keuls multiple comparison or nonparametric tests such as Mann–Whitney test, or Kruskal–Wallis test with post hoc Dunn test were used. *Indicates a p-value <0.05.
CHAPTER 3

Results

Classical monocytes drive atheroprogression

As selective depletion of monocyte subsets is not feasible we developed a novel approach to dissect the specific contribution of monocyte subsets to atherosclerosis (Fig 1A). In Apoe⁻/⁻ mice fed a high-fat diet (HFD) for 4 weeks, the mobilization of leukocytes from the BM was abrogated by application of the cytostatic drug cyclophosphamide (CPM) during subsequent 4 weeks of HFD. This resulted in an absolute leukopenia (Supporting Information Table 1). To address a specific role of monocyte subsets, mice were repeatedly reconstituted with white blood cells from age-matched donor mice, in which either classical or non-classical monocytes were selectively removed by fluorescence activated cell sorting (FACS). Lipid levels, body and spleen weight were not influenced by this regimen (Supporting Information Table 2 and 3). In addition, antibody based depletion of monocyte subsets did not alter white blood cell activation, as assessed by analysis of CD11b expression, CD62L shedding, Annexin V-binding, and reactive oxygen species production (Supporting Information Fig 1). To further validate the transfer efficacy of the reconstitution protocol, we employed the CD45.1/CD45.2 system. The results demonstrate that this protocol allowed for virtual reconstitution of white blood cell subsets (Supporting Information Fig 2) and enabled prominent lesional accumulation of donor leucocytes (Supporting Information Fig 3).

After 8 weeks of HFD, Apoe⁻/⁻ mice displayed atherosclerotic lesion formation in the aortic root and the thoracic aorta, which was significantly diminished by application of CPM for 4 weeks (Fig 1B–D). While reconstitution of leucopenic mice with whole white blood cells from donor mice restored lesion sizes, removal of classical monocytes from donor leucocytes but not non-classical monocytes reduced atherosclerotic lesion area to levels observed in mice receiving CPM only (Fig 1B–D). Immunohistochemical analysis of Mac2⁺ macrophage content in atherosclerotic lesions of the aortic root followed a similar pattern (Fig 1E). When analyzing the number of apoptotic TUNEL⁺ cells in aortic roots, half of which originated from macrophages (Supporting Information Fig 4), we found a significant increase in mice reconstituted with leucocytes depleted of classical monocytes (Fig 1F), which are known to display higher phagocytic capacity and thereby contribute to the clearance of apoptotic cells. Analysis of collagen content by sirius red staining revealed a reduction in
Figure 1. Classical monocytes are decisive during atheroprogession. A. Scheme illustrating the approach to investigate the individual contribution of monocyte subsets to atherosclerotic lesion formation. Male Apoe<sup>−/−</sup> mice 6 weeks of age were fed a HFD for a total of 8 weeks. After 4 weeks of HFD, groups I to IV were treated with i.p. CPM 2x/week. Groups II to IV were reconstituted with leucocytes from Apoe<sup>−/−</sup> donor mice by i.v. injections 2x/week using one donor mouse/recipient each 1 day after CPM treatment. Group II was substituted with all CD45<sup>+</sup> leucocytes, group III received CD45<sup>+</sup> leucocytes without classical monocytes, and group IV was reconstituted with CD45<sup>+</sup> leucocytes without non-classical monocytes, in each case depleted by sorting for classical versus non-classical monocytes. B–D. Assessment of atherosclerotic lesion size after staining aortic root sections (B, C) or aortas (D) with oil-red-O. Representative images and quantification are displayed. E. Quantification of monocyte/macrophage content in aortic roots following Mac2 staining. F. Quantification of TUNEL<sup>+</sup> apoptotic cells. G. Analysis of collagen content by sirius red staining and analysis under polarized light. All data are expressed as mean ± SD. *Denotes significant differences between groups. N=9-12 for each group (One-way ANOVA with Newman–Keuls post hoc test).
aortic collagen content in CPM-treated groups, likely due to decreased collagen synthesis in response to CPM (21). In addition, no differences within the CPM-treated groups were identified (Fig 1G). Taken together, these data reveal a dominant role of classical monocytes in atheroprogression.

mCXCL1 mediates hypercholesterolemia-induced monocytosis

Hypercholesterolemia has been reported to affect counts, phenotype, and function of peripheral blood leukocyte subsets. Increased monocyte counts in mice receiving HFD have been attributed to a selective expansion of the classical monocyte subset (7). In our hands, Apoe<sup>-/-</sup> mice fed a HFD exhibited a leukocytosis comprised of increased counts of neutrophils and classical monocytes (Fig 2A and B).

We next aimed at elucidating mechanisms underlying HFD-induced monocytosis. Prolonged life span, reduced conversion, and enhanced production have been implicated in causing HFD-mediated classical monocytosis (7, 22). Hence, we examined facilitated mobilization as complementary mechanisms of classical monocytosis. The CCR2 ligands CCL2/MCP-1 and CCL7/MCP-3 are crucially involved in the mobilization of classical monocytes from the BM under steady-state and inflammatory conditions (23). However, neither CCL2 nor CCL7 serum levels were found to be increased under HFD (Supporting Information Fig 5). Accordingly, Ccr2<sup>-/-</sup> Apoe<sup>-/-</sup> mice displayed reduced circulating...
classical monocyte counts at steady-state but a significant increase after HFD for 8 weeks by a relative degree similar to that observed in Ccr2+/−Apoe−/− mice (Supporting Information Fig 6), suggesting a mechanism independent of the CCR2 axis to be responsible for HFD-induced monocytosis. Likewise, comparable increases in classical monocyte counts were found in Ccr1−/−Apoe−/−, Ccr5−/−Apoe−/−, and Cx3cr1−/−Apoe−/− mice (Supporting Information Fig 6), indicating a dispensable role of these receptors in HFD-induced classical monocytosis.
Since neutrophil homeostasis in steady-state and during hypercholesterolemia is regulated via the CXCR2-CXCL1 axis (19, 24), we investigated whether this also applies to classical monocytes. We found CXCR2 to be expressed on both monocyte subsets with higher surface levels on classical monocytes (Fig 2C and D). Upon HFD, CXCR2 expression was increased on circulating classical monocytes but remained unaltered on non-classical monocytes (Supporting Information Fig 7). In addition, serum levels of mCXCL1 were significantly elevated in Apoe−/− mice fed a HFD (Fig 2E). Thus, both lines of evidence imply a potential involvement of CXCL1-CXCR2 in the regulation of classical monocytes homeostasis under HFD. The ability of CXCL1 to mobilize classical monocytes was further tested by intravenous injection of rmCXCL1 (Fig 2F). Whereas circulating non-classical monocyte levels remained stable, blood classical monocyte levels increased with concomitant decreases in BM and spleen (Fig 2F), the latter being a recently identified reservoir for monocytes (25). To prove the importance of CXCL1 in HFD-induced monocytosis, we treated Apoe−/− mice on HFD with an antibody to CXCL1 or isotype control. Whereas classical monocyte counts significantly increased in mice treated with isotype control, this was prevented by treatment with the anti-CXCL1 antibody (Fig 2G). In line, numbers of classical monocytes in spleen and BM showed a tendency towards an increase in mice receiving the anti-CXCL1 antibody (Supporting Information Fig 8). Aortic root sections of anti-CXCL1 treated mice displayed smaller lesions (Fig 2H) characterized by lower numbers of lesional classical monocytes and macrophages (Fig 2I). The relevance of CXCL1 in patients was further assessed in plasma of a previously described patient cohort with moderate hypercholesterolemia (20). In these patients, we found increased levels of CXCL1 as compared to controls (Supporting Information Fig 9). Taken together, the increase in CXCL1 levels in conjunction with a differential CXCR2 expression pattern indicates that this chemokine/receptor axis is crucially involved in mediating the HFD-induced mobilization of classical monocytes.

Arterial recruitment of classical monocytes depends on CCR1 and CCR5

Previous studies have suggested pivotal contributions of CCR2 and CX3CR1 to monocyte and macrophage accumulation in atherosclerotic lesions (26, 27). However, leukocyte accumulation at sites of inflammation is regulated
Control of atherogenic classical monocytes

at various levels, namely mobilization from sites of production, recruitment, and life span. Since CCR2 is crucial for monocyte mobilization during inflammation (13), while CX3CR1 confers survival signals in monocytes and plaque macrophages (14), we aimed at discerning such mechanisms from recruitment. Given the dominant role of classical monocytes in atheroprogression established herein, we evaluated the relevance of CCR1, CCR2, CCR5, and CX3CR1 in the recruitment of this subset to atherosclerotic arteries only. Notably, HFD did not alter surface expression of chemokine receptors on classical monocytes, although mRNA levels were up-regulated (Supporting Information Fig 7). In addition, expression of decoy receptors D6 and CXCR7 on the surface of classical monocytes was not affected by HFD (Supporting Information Fig 10). Similarly, expression of CD44, which can serve as CCL5 co-receptor, did not change following HFD (Supporting Information Fig 10). Next, we quantified classical monocyte counts in aortic cell suspensions of Apoe-/-, Apoe-/-Ccr1-/-, Apoe-/-Ccr2-/-, Apoe-/-Ccr5-/-, and Apoe-/-Cx3cr1-/- mice by flow cytometry (Supporting Information Fig 11). Compared to control Apoe-/- mice, the number of classical monocytes was significantly reduced in aortas of atherosclerotic mice lacking CCR1, CCR2, and CCR5 but not of those lacking CX3CR1 both after 4 weeks (Supporting Information Table 4) and 8 weeks (Fig 3A) of HFD. In line with a role in mobilization (13, 23), Apoe-/-Ccr2-/- mice displayed reduced classical monocyte counts in the circulation, whereas no differences were observed in Apoe-/-Ccr1-/-, Apoe-/-Ccr5-/-, and Apoe-/-Cx3cr1-/- mice (Fig 3B and C). To further discriminate between homeostasis and recruitment, we correlated the counts of circulating and aortic classical monocytes. While the two parameters were strongly correlated in Apoe-/-, Apoe-/-Ccr2-/-, and Apoe-/-Cx3cr1-/- mice, no correlation was observed in Apoe-/-Ccr1-/- or Apoe-/-Ccr5-/- mice (Fig 3D), suggesting a recruitment deficit in the latter two strains. Whereas macrophage accumulation was more markedly reduced in Apoe-/-Cx3cr1-/- mice than in Apoe-/-Ccr2-/- and Apoe-/-Ccr5-/- mice at both time points, consistent with a role of CX3CR1 monocytes and in macrophage survival (14), the absence of CCR1 limited macrophage accumulation at early time points but appeared to favor macrophage accumulation at later stages (Supporting Information Table 4).

To corroborate these findings, we performed adoptive transfer experiments using classical monocytes sorted from BM of Apoe-/-, Apoe-/-Ccr1-/-, Apoe-/-Ccr2-/-, Apoe-/-Ccr5-/-, and Apoe-/-Cx3cr1-/- mice. From each donor strain, 106 classical monocytes were labelled with carboxyfluorescein succinimidyl ester (CFSE) as a cell tracker and injected intravenously into Apoe-/- mice that had been on HFD for
CHAPTER 3

Figure 3. Correlating circulating and aortic monocyte counts reveals importance of CCR1 and CCR5 in recruitment of classical monocytes. **A.** Absolute numbers of classical monocytes in aortas of indicated mouse strains after HFD for 8 weeks. **B, C.** Circulating total monocyte **(B)** and classical monocyte **(C)** counts in indicated mouse strains after 8 weeks of HFD, as analyzed by flow cytometry. All data are expressed as mean ± SD. * Denotes significant differences between groups. N=15 for each group (One-way ANOVA with Dunnett post hoc test). **D.** Correlation between aortic and circulating classical monocyte counts in indicated mouse strains after 8 weeks of HFD (Pearson correlation).

Figure 4. CCR1 and CCR5 mediate arterial classical monocyte infiltration. **A, B.** Classical monocytes (10^6) of indicated donor mouse strains were injected into Apoe^−/− recipients after labelling with the cell tracker CFSE and allowed to circulate for 24 h. Both donor mice and recipients had been on HFD for 8 weeks. Gating strategy **(A)** and absolute numbers of labeled cells in the aorta quantified by flow cytometry **(B)** are depicted. * Denotes significant differences compared to injection of classical monocytes from Apoe^−/− donor mice. n=7 for each group (Kruskal–Wallis with Dunns post hoc test). **C, D.** Classical CD45.2^+ monocytes (10^6) of indicated donor mouse strains were injected into CD45.1/Ldlr^−/− recipients and allowed to circulate for 24 h. Both donor mice and recipients had been on HFD for 8 weeks. Gating strategy **(C)** and absolute numbers of CD45.2^+ monocytes in the aorta as quantified by flow cytometry **(D)** are depicted. * Denotes significant differences compared to injection of classical monocytes from Apoe^−/− donor mice. n=7 for each group (Kruskal–Wallis with Dunns post hoc test). **E.** Visualization of leucocyte adhesion to the carotid artery of Apoe^−/−Cx^−/− mice having been on HFD for 8 weeks. To discriminate between classical and non-classical monocytes a PE-conjugated antibody to Gr1 was injected. Scale bar=50 mm. **F.** Quantification of adhesion of classical monocytes to carotid arteries of Apoe^−/−Cx^−/− mice having been on HFD for 8 weeks and having received a single dose of indicated chemokine receptor antagonist 1 h prior to experimentation. All data are expressed as mean ± SD. * Denotes significant differences compared to control mice. n=7–8 (Kruskal–Wallis with Dunns post hoc test) for each group.
Control of atherogenic classical monocytes
8 weeks. After 24 h, adoptively transferred classical monocytes were quantified in aortic homogenates of recipient mice (Fig 4A and B and Supporting Information Fig 12). The recruitment of classical monocytes deficient in CCR1 or CCR5 but not of those deficient in CCR2 or CX3CR1 was severely diminished, when compared to monocytes with intact chemokine receptor profile. To further substantiate these findings, we employed the same strategy but instead used the CD45.1/CD45.2 system to monitor aortic recruitment of classical monocytes. Based on improved discrimination of donor cells within the aortas of CD45.1/ Ldlr-/- recipient mice, the results corroborate the importance of CCR1 and CCR5 for arterial monocyte influx (Fig 4C and D).

This approach was further complemented by intravital microscopy of the carotid artery using Apoe-/- Cx3cr1egfp/+ reporter mice. To specifically track classical monocytes, a PE-conjugated antibody to Gr1 was injected, rendering classical monocytes red/green fluorescent (Fig 4E). The involvement of CCR1, CCR2, CCR5, or CX3CR1 in the adhesion of classical monocytes to carotid arteries of Apoe-/- Cx3cr1egfp/+ mice after 8 weeks of HFD was investigated by intraperitoneal administration of specific antagonists to the respective chemokine receptor 1 h prior to recording. Inhibition of CCR1 or CCR5 markedly reduced luminal adhesion of classical monocytes, an effect not observed by the presence of antagonists to CCR2 or CX3CR1 (Fig 4F). Collectively, these data point at a prevalent function of CCR1 and CCR5 in the recruitment of classical monocytes under acute as well as chronic inflammatory and atherogenic conditions.

Discussion

The importance of monocytes/macrophages in atherosclerosis is widely acknowledged. However, the principal mechanisms of their proatherogenic function, namely differential contributions of monocyte subsets, the control of their homeostasis and recruitment in hypercholesterolemia and atherosclerosis remain insufficiently defined. Herein, we have unequivocally established a predominant role of classical monocytes in atheroprogression. As a major risk factor for atherosclerosis, we found HFD-induced hypercholesterolemia to enhance classical monocytes counts by engaging the mCXCL1-CXCR2 axis. Finally and contrary to previous reports, the CCL5 receptors CCR1 and CCR5 were identified to be essential for the recruitment of classical monocytes to atherosclerotic arteries.
Our data provide several lines of evidence for differential roles of the chemokine receptors CXCR2, CCR1, CCR2, CCR5, and CX3CR1 in arterial monocyte accumulation. Previous studies revealed diminished atherosclerotic plaque formation in mice deficient in these receptors or their respective ligands (9). However, chemokine receptors do not only control monocyte recruitment at sites of inflammation but also their mobilization from the BM, and their life span (9). CXCR2 has been shown to mediate recruitment of monocytes and neutrophils to atherosclerotic arteries (19, 28), in part explaining the reduced atherosclerotic lesion formation in CXCR2-deficient mice. An alternative role of CXCR2 in atherogenesis was unveiled by our findings that its ligand CXCL1 mediates mobilization of classical monocytes under hypercholesterolemia. Under these conditions, CXCL1 may be derived from activated endothelium covering atherosclerotic lesions (29). The importance of CXCL1 in atherosclerotic lesion formation and macrophage accumulation was established by CXCL1 neutralization (28), which may be explained by the influence on monocyte mobilization under HFD identified herein. Elevated mCXCL1 levels under HFD not only impart neutrophilia but also promote mobilization of classical monocytes exhibiting higher CXCR2 surface expression than their non-classical counterparts. Mobilization of classical monocytes along with higher circulating monocyte counts correlate with plaque sizes (7, 26). In conjunction with its role in arterial cell recruitment, the atherogenic effects of CXCR2 may thus be attributable to HFD-mediated effects on monocyte homeostasis.

Whereas various studies in Ccr2−/− mice support an important role of CCR2 in monocyte extravasation, three different approaches employed in this study to discern its effects on homeostasis and recruitment, clearly imply that arterial recruitment of classical monocytes does not require CCR2. Accordingly, several recent studies using adoptive transfer strategies suggest that CCR2 is dispensable for peripheral recruitment of classical monocytes (13, 30). Hence, reduced atherosclerotic lesion sizes in Ccr2−/−Apoe−/− mice (12) may primarily result from lower counts of circulating classical monocytes rather than defects in their recruitment. In contrast to CCR2 and CX3CR1, CCR1 and CCR5 were found to be crucially involved in the recruitment of classical monocytes to atherosclerotic arteries. Both receptors share an overlapping spectrum of ligands among them CCL3 and CCL5, which are present in atherosclerotic lesions through expression or deposition. While global blockade of CCL5 receptors using Met-CCL5 and CCR5 deficiency are associated with reduced atherosclerotic lesion size, CCR1-
deficiency (somatic or in BM) was shown to exacerbate plaque formation (15).

Notably, the effects of plaque development in Ccr1−/− mice appear to be highly stage dependent. When compared to control mice, atherosclerotic lesions in Ccr1−/−Apoe−/− mice are smaller after 1 month of HFD, comparable at 2 months and larger at 3 months of HFD. Whereas early-stage effects may be due to decreased recruitment of neutrophils (19) or monocytes, the exacerbation at later stages may reflect effects favoring macrophage accumulation or a stimulation of T-cell-driven immune responses (15). In contrast, findings for CCR5 are much clearer, i.e. mice deficient in CCR5 exhibit smaller lesions with reduced numbers of mononuclear cells in several models (15). The non-redundant importance of both CCR1 and CCR5 identified herein can be explained by a concept proposing a division of labour during the emigration process, where CCR1 mediates monocyte arrest, CCR5 supports monocyte spreading and both contribute to transendothelial migration towards CCL5 (31).

With the emergence of two monocyte subsets, a multistep model would envision that the relative surface expression of adhesion molecules and chemokine receptors determines the recruitment behavior of each subset. Hence, higher CCR1 levels on classical monocytes may reflect its relevance for the recruitment of this monocyte subset, whereas CCR5 is equally expressed on both subsets, thus partially explaining an involvement in the rare recruitment of non-classical monocytes (27). The prominent role of CCR1 and CCR5 can also be related to the deposition of platelet-derived chemokines mediating proatherogenic monocyte adhesion on endothelium (32). Namely, the CCR1 and CCR5 agonist CCL5 triggers monocyte arrest, an effect further enhanced when CCL5 interacts with CXCL4 (33). The in vivo relevance of this synergistic interaction was substantiated by findings that disruption of CCL5-CXCL4 heteromer formation markedly inhibited atherosclerotic lesion formation (33). In addition, platelet-derived CCL5 can promote arterial recruitment of neutrophils via engagement of CCR1 and CCR5 (19). By release and deposition of granule contents, neutrophils specifically induce adhesion and recruitment of classical monocytes (34-36). Hence, the prominent role of CCR1 and CCR5 may in part reflect the contribution of platelet- and neutrophil-borne proteins with recruitment activity.

While previous data have suggested a function of CX3CR1 in the recruitment of classical monocytes (27) despite lower expression than in non-classical monocytes, recent data offer an alternative explanation (14).
Control of atherogenic classical monocytes

Beyond recruitment, the CX₃CL1-CX₃CR1 axis confers essential survival signals for monocytes, whereas its absence leads to increased death of plaque monocytes and foam cells, providing a mechanism for reduced plaque sizes of CX₃CR1- deficient mice (27; 37). Accordingly, our data indicate that CX₃CR1 is dispensable or redundant in the arterial recruitment of classical monocytes but that its role in arterial recruitment of non-classical monocytes merits further investigation.

A group of silent or decoy receptors able to sequester chemokines (38) may also be important for monocyte trafficking in atherosclerosis. D6, an important member of the decoy receptor family, binds a broad range of CCR1, CCR2, and CCR5 ligands, prevents excessive infiltration of classical monocytes and neutrophils into the myocardium in a mouse model of myocardial infarction (39). In addition, CXCR7 acts as a scavenger for CXCL12, a chemokine important in the retention of stem cells and neutrophils in the BM (40, 41). The relevance of chemokine-scavenging receptors to atherogenic mobilization and recruitment of myeloid cell subsets remains to be investigated. Arrest chemokines, such as CXCL2, CXCL8, and CCL5 (alone or as heterodimer with CXCL4) bind to the glycosaminoglycans on the surface of endothelial cells, immobilize, and mediate firm adhesion of rolling monocytes. The association of chemokines with heparin sulphate can immobilize chemokines on the vessel wall to provide strong and localized signals for integrin activation (42). The interaction of chemokines with glycosaminoglycans or heparan sulphate-decorated CD44 may strengthen chemokine function by various mechanisms, including induction of conformational changes with enhanced activity, protection from proteolytic inactivation, and induction of dimer or heteromer formation (43). Notably, the expression of D6, CXCR7, and CD44 shaping chemokine function was unaltered by HFD in our model. In conclusion, our data establish the quintessential impact of classical monocytes on atheroprogression. Our findings further identify sequential contributions of the CXCL1/CXCR2 axis in the proatherogenic mobilization of classical monocytes and of the CCL5 receptors CCR1 and CCR5 in the control of their recruitment to atherosclerotic arteries. In addition, further experimentation is needed to investigate to what extend chemokines control post-recruitment processes contributing to lesional macrophage accumulation.
Acknowledgements

The authors wish to acknowledge Xhina Balaj, Melanie Garbe, Yvonne Jansen, Patricia Lemnitzer, Silvia Roubrocks and Stefanie Wilbertz for excellent technical assistance.

Source of funding

This study was supported by Deutsche Forschungsgemeinschaft (SFB914 TP B08, SO876/3-1, ZE827/1-1, ZE827/4-1, FOR809), NWO (VIDI 91712503 to O.S., VICI 918.10.616 to C.W.), European Research Council AdG N8249929 (to C.W.), DZHK (MHA VD1.2), German Heart Foundation and Leducq Transatlantic Network of Excellence CVGeneF(x).
References


91
CHAPTER 3


Supporting Information

Supporting Information Figure 1: Antibody-based monocyte selection does not activate leukocytes. Leukocytes were incubated with an antibody cocktail (anti-CD45, anti-CD115, anti-Gr1, referred to as ‘positive selection’) or left untreated (referred to as ‘untouched’) and white blood cells were subsequently FACS-sorted. A: Display of FSC vs. SSC of leukocyte subpopulations after phenotypic identification based on SSC, anti-CD11b, anti-Ly6C, and anti-CD3 staining. B: Analysis of CD11b (left) and CD62L (right) surface expression on leukocyte subsets. C: Apoptosis analysis as assessed by Annexin V binding properties. D: Reactive oxygen species production in myeloid cell subsets in response to PMA (20 nM). All data are expressed as mean ± SD. n = 6 for each group (Students t-test).
Supporting Information Figure 2: Validation of white blood cell reconstitution.

A: Efficiency of FACS-sorted depletion of monocyte subpopulations. White blood cells from CD45.2 mice were harvested (top row) and left intact (bottom left), depleted of classical (bottom middle), or depleted of non-classical monocytes (bottom right).

B: Reconstitution of myeloid cell subsets. Identification of CD45.1-CD45.2^+CD11b^+ donor myeloid cells in recipient mice (top row). Recipient mice were injected with white blood cells (bottom left), or white blood cells depleted of classical monocytes (bottom middle) or depleted of nonclassical monocytes (bottom right). Frequencies of CD45.1-CD45.2^+CD11b^+ parent gate are displayed for each quadrant.
Supporting Information Figure 3: Adoptively transferred leukocytes accumulate in atherosclerotic lesions. Male CD45.1 LDLr⁻/⁻ mice 6 weeks of age were fed a high-fat diet (HFD) for a total of 8 weeks. After 4 weeks of HFD mice were treated with cyclophosphamide (CPM) 2x/week. Mice were reconstituted with leukocytes from CD45.2 ApoE⁻/⁻ donor mice by i.v. injections 2x/week using one donor mouse/recipient each 1 day after CPM treatment. **A:** Representative FACS charts displaying accumulation of CD45.2 monocytic cells in the aortas of CD45.1 LDLr⁻/⁻ mice. **B:** CD45.1- and CD45.2-positive leukocytes were identified in aortic root sections of recipient mice. Scale bar indicates 50μm left/right group of pictures and 100μm in the central group.
Supporting Information Figure 4: Apoptotic cells are partially of macrophage origin. Aortic root sections were stained with TUNEL and anti-Mac2 antibodies. Cells positive for TUNEL and Mac2, white arrows; cells positive for TUNEL only, brown arrows. Scale bar is 50 μm.

Supporting Information Figure 5: CCL2/CCL7 serum levels are unaltered by hypercholesterolemia. Concentrations of serum CCL2 and CCL7 of Apoe⁻/⁻ mice fed a chow diet or high-fat diet (HFD) for 8 weeks were quantified by ELISA. All data are expressed as mean ± SD. n = 8-11 for each group (Students t-test).

Supporting Information Figure 6: Hypercholesterolemia-induced monocytosis is independent of CCR1, CCR2, CCR5, and CX₃CR1. Classical monocytes were quantified in indicated mouse strains fed a chow diet or high-fat diet (HFD) for 8 weeks. All data are expressed as mean ± SD. * denotes significant differences between groups. n = 8-9 for each group (Students t-test).
Supporting Information Figure 7: Effect of hypercholesterolemia on chemokine receptor surface expression on classical monocytes. A: Transcriptional expression changes of indicated chemokine receptors in classical monocytes of Apoe<sup>−/−</sup> mice fed a chow diet or high-fat diet (HFD) for 8 weeks were assessed by PCR array studies. B: Expression of CCR1, CCR2, CCR5, CXCR1, and CXCR2 (mean fluorescence intensity, MFI) on classical monocytes from Apoe<sup>−/−</sup> mice fed normal chow or HFD for 8 weeks were assessed by FACS. All data are expressed as mean ± SD. * indicates significant difference between groups. n = 3- 5 for each group (Mann-Whitney U-test).

Supporting Information Figure 8: Effect of CXCL1-neutralization on classical monocytes in bone marrow and spleen. Apoe<sup>−/−</sup> mice were fed high fat diet for 4 weeks and injected (5μg i.p., daily during first week, 3x/week in subsequent weeks) with isotype control or an antibody to mCXCL1. Displayed are absolute counts of classical monocytes in the bone marrow (left) or spleen (right), as assessed by flow cytometry. All data are expressed as mean ± SD. n=8 for each group.
Supporting Information Figure 9: Plasma CXCL1 levels are increased in patients with moderate hypercholesterolemia. Plasma CXCL1 was assessed in patients with moderate hypercholesterolemia by ELISA. n = 10 for each bar (Students t-test). * denotes significant differences between groups.

Supporting Information Figure 10: High-fat diet does not alter expression of D6, CXCR7, or CD44 on classical monocytes. Apoe<sup>−/−</sup> mice were fed a high-fat diet (HFD) for 4 weeks or received chow diet. Expression of CXCR7, CD44, and D6 on classical monocytes was measured by flow cytometry. Displayed are representative histograms and quantification. All data are expressed as mean ± SD. n = 4 for each group. FMO, fluorescence minus one; sMFI, specific mean fluorescence intensity.
CHAPTER 3

Supporting Information Figure 11: Gating strategy for detection of classical monocytes in aortas. Shown are representative dot blots and gates.

Supporting Information Figure 12: Aortic recruitment of adoptively transferred classical monocytes depends on CCR1 and CCR5. Classical monocytes (10^6) isolated from the bone marrow of indicated donor mouse strains by FACS sorting were injected into Apoe^−/− recipients after labeling with the cell tracker CFSE and allowed to circulate for 24 hours. Both donor mice and recipients had been on high-fat diet (HFD) for 8 weeks. Number of CFSE^+ cells in the aorta is expressed in % of CD45^+ cells. * denotes significant differences compared to injection of classical monocytes from Apoe^−/− donor ice. n = 7 for each group (Kruskal-Wallis with Dunns post-hoc test).
Supporting Information Table 1: Repeated cyclophosphamide-injection induces severe leukopenia. Injection of 100 mg/kg cyclophosphamide (CPM) two times a week leads to significantly reduced circulating leukocyte subpopulations. * indicates significant difference compared to respective controls. n = 6 for each group (Mann-Whitney U-test).

<table>
<thead>
<tr>
<th></th>
<th>granulocytes</th>
<th>monocytes</th>
<th>classical monocytes</th>
<th>nonclassical monocytes</th>
<th>T-cells</th>
<th>B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.3 x 10^5</td>
<td>2.5 x 10^5</td>
<td>1.8 x 10^5</td>
<td>0.7 x 10^5</td>
<td>19.7 x 10^5</td>
<td>37.2 x 10^5</td>
</tr>
<tr>
<td></td>
<td>+/- 1.5 x 10^5</td>
<td>+/- 0.4 x 10^5</td>
<td>+/- 0.4 x 10^5</td>
<td>+/- 0.1 x 10^5</td>
<td>+/- 3.1 x 10^5</td>
<td>+/- 8.0 x 10^5</td>
</tr>
<tr>
<td>CPM</td>
<td>0.2 x 10^5*</td>
<td>0.01 x 10^5*</td>
<td>0.1 x 10^5*</td>
<td>0.1 x 10^5*</td>
<td>7.1 x 10^5*</td>
<td>0.7 x 10^5*</td>
</tr>
<tr>
<td></td>
<td>+/- 0.1 x 10^5</td>
<td>+/- 0.1 x 10^5</td>
<td>+/- 0.1 x 10^5</td>
<td>+/- 0.1 x 10^5</td>
<td>+/- 1.3 x 10^5</td>
<td>+/- 0.4 x 10^5</td>
</tr>
</tbody>
</table>

Supporting Information Table 2: Leukocyte ablation and reconstitution does not alter blood lipid levels. Plasma lipid levels were assessed at the end of the ablation-reconstitution cycle by use of EnzyChrom™ Triglyceride Assay Kit and the EnzyChrom™ HDL and LDL/VLDL Assay Kit. n = 9-12 for each group.

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides</th>
<th>Total Cholesterol</th>
<th>LDL/VLDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 0 (HFD only)</td>
<td>179.1 +/- 57.9</td>
<td>539.9 +/- 59.7</td>
<td>264.4 +/- 77.8</td>
<td>189.8 +/- 74.9</td>
</tr>
<tr>
<td>Group I (HFD + CPM injection)</td>
<td>184.3 +/- 54.17</td>
<td>516.3 +/- 69.02</td>
<td>238.9 +/- 52.3</td>
<td>158.4 +/- 74.4</td>
</tr>
<tr>
<td>Group II (HFD + CPM injection + reconstituted with WBC)</td>
<td>201.1 +/- 40.8</td>
<td>490.8 +/- 58.3</td>
<td>180.3 +/- 68.1</td>
<td>142.0 +/- 29.1</td>
</tr>
<tr>
<td>Group III (HFD + CPM injection + reconstituted with WBC)</td>
<td>208.5 +/- 56.17</td>
<td>489.4 +/- 80.4</td>
<td>218.8 +/- 74.2</td>
<td>131.5 +/- 30.1</td>
</tr>
<tr>
<td>Group IV (HFD + CPM injection + reconstituted with WBC - non-classical monocytes)</td>
<td>188.0 +/- 68.6</td>
<td>506.7 +/- 89.03</td>
<td>198.8 +/- 87.9</td>
<td>169.0 +/- 72.77</td>
</tr>
</tbody>
</table>
Supporting Information Table 3: Leukocyte ablation and reconstitution does not alter body or spleen weights. Body and spleen weights were assessed before initiating high-fat diet as well as at the end of the experiment in each group. \( n = 9-12 \) for each group.

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides</th>
<th>Total Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before HFD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.6 +/- 0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Group 0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HFD only)</td>
<td>30.2 +/- 2.0</td>
<td>71.5 +/- 10.9</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HFD + CPM injection)</td>
<td>27.4 +/- 2.1</td>
<td>84.8 +/- 22.7</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HFD + CPM injection + reconstituted with WBC)</td>
<td>27.6 +/- 1.1</td>
<td>105.0 +/- 25.5</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HFD + CPM injection + reconstituted with WBC – classical monocytes)</td>
<td>28.00 +/- 1.9</td>
<td>108.0 +/- 15.7</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HFD + CPM injection + reconstituted with WBC – non-classical monocytes)</td>
<td>27.2 +/- 2.0</td>
<td>111.9 +/- 14.7</td>
</tr>
</tbody>
</table>
Supporting Information Table 4: Accumulation of atherosclerotic lesion sizes, monocytic cell counts in the blood and in the aorta, and correlation thereof after 4 and 8 weeks of HFD. All data are expressed as mean ± SD. n = 5-7 for mice fed a HFD for 4 weeks and n = 13-15 for mice fed a HFD for 8 weeks. * indicates significant difference compared to respective Apoe^{-/-} mice. HFD, high-fat diet; CM, classical monocytes; NCM, non-classical monocytes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Oil-red O+ area (% of aortic root area)</th>
<th>total circulating monocyte count (x10^9/ml)</th>
<th>circulating CM count (x10^5/ml)</th>
<th>CM number/aorta</th>
<th>Correlation circulating CM/aortic CM</th>
<th>Macrophage number/aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe^{-/-}</td>
<td>8.4 ± 1.9</td>
<td>5.8 ± 1.7</td>
<td>3.7 ± 1.2</td>
<td>187.5 ± 47.5</td>
<td>r = 0.8855 p = 0.0457</td>
<td>17723</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>3.8* ± 1.2</td>
<td>5.8 ± 2.2</td>
<td>4.3 ± 1.7</td>
<td>100.3* ± 18.8</td>
<td>r = -0.6426 p = 0.5557</td>
<td>4113*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>3.3* ± 1.9</td>
<td>1.2* ± 0.2</td>
<td>0.4* ± 0.1</td>
<td>84.2* ± 45.5</td>
<td>r = 0.9570 p = 0.0430</td>
<td>8230*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>3.6* ± 1.2</td>
<td>6.5 ± 1.2</td>
<td>4.4 ± 0.8</td>
<td>114.0* ± 13.6</td>
<td>r = 0.0109 p = 0.9890</td>
<td>9554*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>2.8* ± 1.8</td>
<td>4.5 ± 2.0</td>
<td>3.4 ± 1.5</td>
<td>162.0 ± 92.9</td>
<td>r = 0.9152 p = 0.0293</td>
<td>4567*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>12.0 ± 3.1</td>
<td>4.9 ± 2.1</td>
<td>3.0 ± 1.2</td>
<td>178.8 ± 74.6</td>
<td>r = 0.8777 p = 0.0001</td>
<td>18738</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>5.5* ± 2.4</td>
<td>0.9* ± 0.5</td>
<td>0.3* ± 0.2</td>
<td>48.2* ± 22.9</td>
<td>r = 0.8885 p = 0.0001</td>
<td>11551*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>6.6* ± 2.7</td>
<td>4.0 ± 1.7</td>
<td>2.9 ± 1.2</td>
<td>102.4* ± 30.5</td>
<td>r = -0.1003 p = 0.7221</td>
<td>10389*</td>
</tr>
<tr>
<td>Apoe^{-/-}</td>
<td>4.4* ± 2.5</td>
<td>3.5 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>148.1 ± 56.19</td>
<td>r = 0.8048 p = 0.0003</td>
<td>7542*</td>
</tr>
</tbody>
</table>