Chemokine Receptor 7 Knockout Attenuates Atherosclerotic Plaque Development

Maren Luchtefeld, PhD; Christina Grothusen, MD; Andreas Galalick, MD; Kumaravelu J. Jagavelu, PhD; Harald Schuett, MD; Uwe J. F. Tietje, MD, PhD; Oliver Pabst, PhD; Karsten Grote, PhD; Helmut Drexler, MD†; Reinhold Förster, MD; Bernhard Schieffer, MD

Background—Atherosclerosis is a systemic inflammatory disease characterized by the formation of atherosclerotic plaques. Both innate immunity and adaptive immunity contribute to atherogenesis, but the mode of interaction is poorly understood. Chemokine receptor 7 (CCR7) is critically involved in the transition from innate to adaptive immune activation by coordinating the migration to and positioning of antigen-presenting dendritic cells and T cells in secondary lymphoid organs. More recently, it was shown that CCR7 is also responsible for T-cell migration into inflamed tissues and T-cell egress from these tissues via the afferent lymph. Thus, we investigated the influence of a systemic CCR7 deficiency on atherogenesis in atherosclerosis-prone low-density lipoprotein receptor (ldlr) knockout mice.

Methods and Results—CCR7 deficiency resulted in reduced atherosclerotic plaque development. CCR7−/− T cells showed impaired entry and exit behavior from atherosclerotic lesions. Oxidized low-density lipoprotein, a key molecule for atherogenesis with antigenic features, was used to pulse dendritic cells and to expand T cells ex vivo. Adoptive transfer of C57BL/6 wild-type T cells but not ccr7−/−-derived T cells primed with oxidized low-density lipoprotein–pulsed dendritic cells resulted in a reconstitution of atherogenesis in ccr7−/−/ldlr−/− mice.

Conclusion—These results demonstrate that both CCR7-dependent T-cell priming in secondary lymphoid organs and CCR7-dependent recirculation of T cells between secondary lymphoid organs and inflamed tissue are crucially involved in atherosclerotic plaque development. (Circulation. 2010;122:1621-1628.)

Key Words: atherosclerosis ■ lipids ■ immune system ■ lymphocytes

Atherosclerosis is a chronic systemic inflammatory disease characterized by the accumulation of lipids in the arterial wall, frequently leading to myocardial infarction, stroke, and sudden death.1 In early atherogenesis, monocytes are recruited into the arterial vessel wall, where they differentiate into macrophages, take up cholesterol deposits (mainly oxidized low-density lipoproteins [oxLDL]), become foam cells, and form the initial atherosclerotic lesion. At the same time, secretion of inflammatory cytokines results in further recruitment of monocytes, T cells, and cells of the vessel wall into the lesion, thereby driving atherosclerotic plaque growth and maturation. Although there is profound evidence for the importance of both innate and adaptive immunity for atherosclerotic plaque development,2 the transition processes and mode of interaction between these immune responses remain unclear. However, accumulating evidence indicates that oxLDL may represent a connecting piece between innate and adaptive immunity in this setting. In this regard, different groups were able to show that stimulation of oxLDL led not only to dendritic cell (DC) maturation3,4 but also to clonal T-cell proliferation and differentiation into proinflammatory T helper (Th) 1 cells.5,6

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The classic activation of adaptive immunity takes place in secondary lymphoid organs (SLOs) where antigen presentation by DCs to naïve T cells results in activation and functional differentiation of these T cells. The C-C-chemokine receptor type 7 (CCR7) has been described to be crucially involved in several fundamental processes shaping the structural and functional organization of the adaptive immune system.7 CCR7 is regarded as a key receptor in the coordinated migration of naïve T cells and matured DCs to and their localization within SLOs. Subsequently, CCR7 deficiency results in impaired homing and positioning of T-cell and DC populations within SLOs, thereby leading to a...
severely delayed induction of adaptive immune responses and impairment of central and peripheral tolerance. However, CCR7 is expressed not only on naive T cells and matured DCs but also on antigen experienced memory T cells,8,9 allowing their migration into tissues expressing the CCR7 ligands CCL19 and 21.

In this regard, recent studies suggested that the enhanced endothelial expression of CCR7 ligands in chronically inflamed organs10,11 or within atherosclerotic lesions3,12 causes sustained T-cell recruitment to sites of inflammation.13,14 Moreover, because CCR7 deficiency results in accumulation of T cells in inflamed tissues,15 T-cell egress from sites of inflammation into draining lymph nodes also depends on CCR7.

Figure 1. Analysis of atherosclerotic plaque development after eating a high-cholesterol diet for 12 weeks. A, En face preparation of aortas (left) and aortic roots (right) stained with Oil Red O revealed reduced atherosclerotic plaque development in ccr7−/−/ldlr−/− mice (CCR7−/−) compared with ldlr−/− mice (CCR7+/+). B, Quantification of Oil Red O−stained areas as a percentage of whole aortic area (n=8 to 9 animals per group; *P<0.05 vs ldlr−/− mice) or as a total lesion area (mm2) in the aortic root (n=6 to 12 animals per group; 3 to 5 sections per animal; *P<0.05 vs ldlr−/− mice; ×100 magnification). C, Left, A representative immunohistochemical staining of macrophage-marker MOMA-2 shows more macrophages within atherosclerotic lesions of ldlr−/− mice (CCR7−/−) (×100 magnification). Right, Quantification of MOMA-2 positively stained area as percentage of total plaque area (n=7 to 9 animals per group; 3 to 5 sections per animal; mean±SEM; Student t test, *P=0.02 vs ldlr−/− mice). D, top, A representative immunohistochemical staining of CD11c+ cells in the aortic root demonstrates more DCs within atherosclerotic lesions of ccr7−/−/ldlr−/− mice compared with ldlr−/− mice (×200 magnification). Bottom, Quantification of CD11c+−stained area as percentage of total plaque area (n=15 animals per group; 2 to 4 sections per animal; mean±SEM; Student t test; *P=0.038 vs ldlr−/−). E, top, A representative immunohistochemical staining of T cells (CD3, left; CD4, right) reveals more CD3+ and CD4+ cells within aortic roots of ccr7−/−/ldlr−/− mice compared with ldlr−/− mice (×200 magnification). Bottom, Quantification of CD3+ and CD4+ cells within the aortic roots of ldlr−/− and ccr7−/−/ldlr−/− mice. CD3+ (left; mean±SEM; Student t test, *P=0.032 vs ldlr−/− mice) and CD4+ (right; median with 25th and 75th percentiles; Mann-Whitney rank-sum test, *P=0.002 vs ldlr−/− mice) cells were counted directly (n=12 to 18 animals per group; 6 to 9 sections per animal). F, Quantification of CD11c+ cells as a percentage of all CD45+ cells within the MLN of ldlr−/− and ccr7−/−/ldlr−/− mice (n=6 animals per group; Student t test, *P=0.031 vs ccr7−/−/ldlr−/− mice). G, Representative immunofluorescence staining of T cells of CCR7−/− mice and CCR7−/− mice (anti-CD3, red) and B cells (anti-B220, blue) in MLNs (n=4 to 5 per group). Bar=200 μm.

Given the importance of CCR7 for facilitating the transition from innate to adaptive immune activation and its general role in T-cell migration, we investigated the impact of a systemic CCR7 deficiency on atherogenesis.

Methods

Animals

CCR7-deficient mice (C57BL/6) were crossed with LDL receptor–deficient mice (C57BL/6, Jackson Laboratories, Bar Harbor, Me) to obtain double-knockout mice (ccr7−/−/ldlr−/−). Littermates heterozygous for CCR7 (ccr7+/−/ldlr−/−) or homozygous for the wild-type (WT) allele (ccr7−/−/ldlr−/−) served as controls. All mice were kept under specific pathogen free conditions. All experiments were approved by the government animal ethics committee and carried out...
according to the guidelines of the Federation of European Animal Science Associations. At 10 weeks of age, male mice were subjected to a high-cholesterol diet (product D12108, Research Diets; 1.25% cholesterol without cholate (Brogaarden, Gentofte, Denmark) for 12 weeks. Subsequently, mice were euthanized, and the aortas were removed and analyzed as described below. Aortic arches were frozen in optimal-cutting-temperature compound (Tissue-Tek, Mainz, Germany).

Statistical Analysis

Data are presented as mean±SD or SEM when normally distributed. When data did not pass the test for normality, they are presented as median with 25th and 75th percentiles. Comparisons between 2 groups were performed by the Student t test when data were normally distributed. The Mann-Whitney rank-sum test was used when data were not normally distributed or if group variances were unequal. One-way ANOVA was used for comparisons between ≥3 groups followed by the Holm-Sidak method for multiple pairwise comparisons when data were normally distributed. The Kruskal-Wallis test followed by the Dunn posthoc test were used when group data were not normally distributed or if group variances were unequal. P values <0.05 were considered statistically significant. An expanded Material and Method section is available in the online-only Data Supplement.

Results

CCR7 Deficiency Reduces Atherosclerotic Plaque Development

To investigate the impact of CCR7 on atherogenesis, atherosclerosis-prone ldlr−/− mice were crossed with ccr7−/− mice. Atherosclerotic plaque development was analyzed after feeding 10-week-old male mice a high-cholesterol diet for 12 weeks. Aortic lipid deposition as a measure for plaque extent was assessed by en face preparation and subsequent Oil Red O staining (Figure 1A, left). The atherosclerotic plaque area was reduced by >50% after 12 weeks of feeding (8.41±3.29% in ldr−/− mice versus 3.63±1.25% in ccr7−/− ldr−/− mice; mean±SD; P<0.05, Figure 1B, left). Additionally, total lesion size in aortic root sections was analyzed after Oil Red O staining (Figure 1A, right); we also observed markedly reduced plaque formation (1.1±0.05 mm² in ldr−/− mice versus 0.81±0.06 mm² in ccr7−/−/ldr−/− mice; mean±SEM; P<0.05; Figure 1B, right).

CCR7 Deficiency Has No Impact on Lipoprotein Fractions or Body Weight

To determine whether differences in lipid metabolism could account for the decrease in atherosclerotic plaque development in ccr7−/−/ldr−/− mice, serum lipids were determined in plasma samples after a 12-hour fasting period. Levels of total cholesterol and high-density lipoprotein (HDL), LDL, and very low-density lipoprotein cholesterol fractions did not differ between the experimental groups (Table 1). Body weights before and after feeding did not differ between genotypes (Table 2).

CCR7 Deficiency Reduces Macrophage Accumulation in Atherosclerotic Plaques

Because macrophage recruitment into the vessel wall and subsequent formation of foam cells are hallmarks of atherosclerotic plaque development, we elucidated the role of CCR7 deficiency on macrophage content of aortic plaques by immunohistochemistry using a monococyte/macrophage stain-

| Table 1. Plasma Levels of High-Cholesterol Diet–Treated Mice |
|-----------------|------------|----------------|------------------|------------------|
| ldlr−/− | ccr7+/−/ldlr−/− | ccr7−/−/ldlr−/− |
| TC, mg/dL | 851±95 | 1151±106 | 935±181 |
| Triglycerides, mg/dL | 150±39 | 212±28 | 248±71 |
| VLDL cholesterol, mg/dL | 327±34 | 413±27 | 317±54 |
| LDL cholesterol, mg/dL | 415±55 | 591±73 | 489±127 |
| HDL cholesterol, mg/dL | 106±4 | 147±12 | 129±5 |

VLDL indicates very low-density lipoprotein. Plasma lipids levels after 12 weeks on a high-cholesterol diet are not significantly different between the experimental groups (mean±SEM; n=7 per group).

CCR7 Deficiency Increases Infiltration of DCs and T Cells in Atherosclerotic Plaques

To identify DCs within atherosclerotic lesions, immunohistochemical analyses were performed. We observed a small but statistically significant increase in CD11c or CD11c+ cells in the aortas of ccr7−/−/ldlr−/− mice (12.78±1.25% in ccr7−/−/ ldlr−/− mice versus 9.3±1.01% in ldr−/− mice; mean±SEM; P=0.038; Figure 1D). We also analyzed the presence of CD3+ and CD4+ T cells within the aortic root by immunohistochemistry. Ccr7−/−/ldr−/− mice showed ~25% more CD3+ cells (87.42±7.60 cells per section in ccr7−/−/ ldr−/− mice versus 65.71±4.89 cells per section in ldr−/− mice; mean±SEM; P=0.032; Figure 1E, left) and ~50% more CD4+ cells within the aortic root compared with ldr−/− mice (82 [25th and 75th percentiles, 43.3 and 113.9] cells per section in ccr7−/−/ldlr−/− mice versus 34.3 [25th and 75th percentiles, 26.8 and 41.9] cells per section in ldr−/− mice; median; P=0.002; Figure 1E, right). These cells were located mainly within the adventitia as previously described by others.16 Given the fact that CCR7 is critically involved in homing of naïve T cells and antigen-presenting DCs to SLOs, we also analyzed the presence of DCs and T cells in SLOs of ccr7−/−/ldlr−/− and ldr−/− mice. As expected, we observed significantly fewer DCs and T cells in the SLOs of ccr7−/−/ ldr−/− mice compared with ldr−/− mice (Figure 1F and 1G). It may be argued that the analysis of lymph nodes more adjacent to the aorta reflects the traveling of T cells and DCs from atherosclerotic lesions more accurately. However, atherosclerosis is a systemic disease and oxLDL is a systematically detectable molecule,17–19 so uptake of oxLDL by antigen-presenting cells may not be locally restricted. In
addition, a high-cholesterol diet administered orally may result in oxLDL formation already during digestion, so mesenteric lymph nodes (MLNs) may actually be one of the first SLOs facilitating contact of oxLDL-pulsed DCs with naïve T cells. Finally, MLNs drain the dominant part of the gastrointestinal organ system and thus may reflect the behavior of DCs and T cells in a large area of arterial vasculature.

CCR7 Deficiency Alters T-Cell Response

To investigate T helper cell responses in *ccr7<sup>-/-</sup>/ldlr<sup>-/-</sup>* mice, T-cell cytokine analyses were performed. Although Th1 cells have been ascribed a proatherogenic role mainly because of the secretion of interferon-γ (INFγ) by Th2 cells is considered atheroprotective, although the function of interleukin-4 (IL-4) plasma levels as determined by ELISA. Analyses were performed in triplicate (n=4 to 5 animals per group; mean±SEM; Student t test, *P=0.027). B, Interleukin-4 (IL-4) plasma levels as determined with 25th and 75th percentiles; Mann-Whitney rank-sum test, *P=0.29).

Figure 2. Analysis of Th1 and Th2 signature cytokines under a cholesterol-rich diet. Th1 but not Th2 response is altered in CCR7<sup>-/-</sup> mice under a cholesterol-rich diet. A, INFγ plasma levels as determined by ELISA. Analyses were performed in triplicate (n=4 to 5 animals per group; mean±SEM; Student t test, *P=0.027). B, Interleukin-4 (IL-4) plasma levels as determined by ELISA. Analyses were performed in triplicate (n=4 to 5 animals per group; mean±SEM; Student t test, *P=0.29).

OxLDL-Pulsed DCs Upregulate CCR7 and Migrate Toward CCL19 In Vitro

Because of the substantial impact of oxLDL as a key molecule for atherogenesis with antigenic features, we stimulated immature WT DCs with oxLDL and investigated DC maturation. We observed that oxLDL- or lipopolysaccharide-treated DCs exhibited the morphology of mature DCs, whereas native LDL (nLDL)–loaded DCs remained morphologically immature (Figure 3A). Using real-time polymerase chain reaction, we observed that oxLDL but not nLDL induced a significant increase in CCR7 and myosin heavy chain-II (MHC-II) mRNA expression on DCs when normalized to β-actin after 24 hours of stimulation (median with 25th and 75th percentiles; *P<0.05; Figure 3B, left). Fluorescence activated cell sorting (FACS) analysis also demonstrated an increase in CCR7/MHC-II double-positive DCs on stimulation with oxLDL (Figure 3B, right). In accordance with the increased expression of CCR7, only oxLDL-pulsed DCs migrated toward the CCR7 ligand CCL19 (median with 25th and 75th percentiles; *P<0.05; Figure 1A in the online-only Data Supplement) in a CCR7-dependent manner (me-
be significantly decreased in **ldlr** after eating a cholesterol-rich diet for 3 months (CCR7/H11002/H110210.05 vs control **P**).

The rate of T-cell proliferation was significantly enhanced after cocultivation with oxLDL (10 and 50 μg/mL) -pulsed DCs compared with T cells cocultivated with unstimulated DCs (control) (mean ± SEM; **P** < 0.05 versus control; Figure 3C). The oxLDL-loaded DCs, we investigated whether these cells are able to induce the proliferation of naïve T cells. Indeed, the oxLDL-specific T-cell receptors after antigen-presentation assay revealed that oxLDL-loaded but not lipopolysaccharide-activated DCs caused an expansion of T cells carrying TCR chain Vβ6 (mean ± SD; **P** < 0.05 versus control). Facs analysis of aortic and mesenteric lymph nodes of **ldlr** increased within the aorta and the mesenteric lymph nodes of **ldlr** mice compared with unstimulated DCs cocultivated with oxLDL-loaded DCs did not restore atherosclerosis in **ldlr** mice versus aorta, and MLN were stained with anti-CD45, and the percentages of CTO- or CTG-positive T cells were determined by FACS analysis. Top left, **CCR7**−/- T-cells; bottom right, WT T cells. Numbers in quadrants are percentages of positive cells. One representative dot plot of 3 independent experiments is shown.

**OxLDL-Pulsed DCs Induce a Specific T-Cell Priming In Vitro**

Because MHC-II gene expression was upregulated on oxLDL-loaded DCs, we investigated whether these cells are able to induce the proliferation of naïve T cells. Indeed, the rate of T-cell proliferation was significantly enhanced after cocultivation with oxLDL (10 and 50 μg/mL) -pulsed DCs compared with T cells cocultivated with unstimulated DCs (control) (mean ± SEM; **P** < 0.05 versus control; Figure 3C).

Next, we determined whether contact with oxLDL-pulsed DCs may result in upregulation of the specific T-cell receptor (TCR) Vβ6, which has previously been identified as a prominent TCR within atherosclerotic lesions.23 We confirmed Vβ6 expression by FACS analysis and observed an oxLDL-specific proliferation of the T-cell clone carrying Vβ6 (mean ± SD; **P** < 0.05 versus control; Figure 4A). Vβ6 expression was also investigated in cell homogenates from aorta and SLOs with FACS analysis. TCR Vβ6 was found to be significantly decreased in **ccr7**−/-/ldlr−/- mice compared with **ldlr**−/- mice (aorta: 15.36% [25th and 75th percentiles, 9.2% and 18.42%] in **ldlr**−/- mice versus 4.05% [25th and 75th percentiles, 3.28% and 5.30%] in **ccr7**−/-/ldlr−/- mice; median; **P** = 0.032; Figure 4B; MLN: 6.85 ± 1.2% in **ldlr**−/- mice versus 3.96 ± 1.84% in **ccr7**−/-/ldlr−/- mice; mean ± SD; **P** = 0.018, Figure 4B, right).

**Figure 4.** Analysis of T-cell receptor expression after cocultivation of T cells with oxLDL-pulsed DCs in vitro. A, FACs analysis of oxLDL-specific T-cell receptors after antigen-presentation assay revealed that oxLDL-loaded but not lipopolysaccharide-activated DCs caused an expansion of T cells carrying TCR chain Vβ6 (mean ± SD; **n** = 4 independent experiments; 1-way ANOVA followed by the Holm-Sidak method, **P** < 0.05 vs control cells). B, FACs analysis of aortic and mesenteric lymphoid CD45+ gated cells revealed that Vβ6+ T cells were significantly increased within the aorta and the mesenteric lymph nodes of **ldlr** mice (CCR7+/−/ldlr−/-) compared with **ccr7**−/-/ldlr−/- mice (CCR7−/-) after eating a cholesterol-rich diet for 3 months (n = 4 to 5 animals per group; aorta: median with 25th and 75th percentiles; Mann-Whitney rank-sum test, **P** = 0.032 vs **ccr7**−/-/ldlr−/- mice; MLN: mean ± SEM; Student t test, **P** = 0.018 vs **ccr7**−/-/ldlr−/- mice).

**Figure 5.** Adoptive transfer of **ccr7**−/-derived T cells and WT T cells into **ldlr**−/- recipient mice. Equal numbers of cell tracker orange (CTO)−/−labeled WT T cells and cell tracker green (CTG)−/−labeled **ccr7**−/-/ldlr−/- derived T cells were injected intravenously into **ldlr**−/- recipient mice on a cholesterol-rich diet. After 48 hours, cell suspensions from aorta, spleen, blood, and MLN were harvested to determine T-cell distribution. As expected, we observed a reduced capacity of **ccr7**−/- T cells to migrate into the MLN compared with WT T cells. In blood samples, **ccr7**−/- T cells were increased compared with WT T cells, whereas within the spleen, we found equal numbers of T cells independently of genotype. T cells from **ccr7**−/- mice displayed a reduced migration into atherosclerotic aortas of recipient mice compared with the migration of WT T cells.

**Injection of WT T Cells but Not **ccr7**−/-Derived T Cells Primed on oxLDL-Pulsed DCs Reverses Impaired Atherosclerotic Plaque Development in **ccr7**−/-/ldlr−/-Deficient Mice**

We hypothesized that the impaired migratory capacity of **CCR7**-deficient DCs to SLOs results in a reduced oxLDL-specific T-cell priming, which is responsible for the decrease in atherosclerotic plaque development in this mouse model. Thus, we speculated that ex vivo T-cell priming with oxLDL-pulsed DCs could restore plaque extent in **ccr7**−/-/ldlr−/- mice. Therefore, we cocultivated WT and **ccr7**−/-/ldlr−/- derived T cells with oxLDL-loaded DCs in vitro as described above and intravenously injected these T cells every 3 weeks into **ccr7**−/-/ldlr−/- mice fed a cholesterol-rich diet for 3 months.

Injection of ex vivo primed WT T cells cocultivated on oxLDL-pulsed DCs induced atherosclerosis in **ccr7**−/-/ldlr−/- mice to an extent comparable to **ldlr**−/- mice (aorta, 9.41% [25th and 75th percentiles, 9.20% and 10.40%]; aortic root total lesion area, 1.03 mm² [25th and 75th percentiles, 0.92 and 1.36 mm²] in **ccr7**−/-/ldlr−/- mice versus aorta, 9.60% [25th and 75th percentiles, 7.43% and 10.81%]; aortic root total lesion area, 1.12 mm² [25th and 75th percentiles, 0.95 and 1.29 mm²] in **ldlr**−/- mice; median; Figure 6A and 6B). In contrast, injection of WT T cells cocultivated with nLDL-loaded DCs or injection of **ccr7**−/-/ldlr−/- derived T cells cocultivated with oxLDL-loaded DCs did not restore athero-
sclerotic plaque development (WT T cells cocultivated with nLDL-loaded DCs: aorta, 7.50% [25th and 75th percentiles, 5.08% and 7.93%]; aortic root total lesion area, 0.86 mm² [25th and 75th percentiles, 0.83 and 0.92 mm²]; CCR7−/−-derived T cells cocultivated with oxLDL loaded DCs: aorta, 4.60% [25th and 75th percentiles, 3.38% and 5.44%]; aortic root total lesion area, 0.38 mm² [25th and 75th percentiles, 0.23 and 0.69 mm²]; median; P<0.05; Figure 6A and 6B). In line with the reduced extent of atherosclerosis, macrophage recruitment into aortic plaques was also enhanced in ccr7−/− mice injected with oxLDL- but not with nLDL-primed T cells or oxLDL-primed CCR7−/−-derived T cells (17.21±1.34%, oxLDL injected; 12.96±1.39%, nLDL injected; 8.31±2.02%, oxLDL-primed CCR7−/−-derived T cells; mean±SEM; P<0.05; Figure 6C and 6D).

Discussion

Activation of the innate immune system has long been recognized as fundamentally involved in atherosclerotic plaque development. More recently, evidence has been provided that the adaptive immunity is also causally involved in the initiation and progression of atherosclerosis. However, both the interaction between innate and adaptive immunity and the true nature of adaptive immune responses in atherosclerosis are only partially characterized.

CCR7 is critical for directing the migration to and the positioning and interaction between naïve T cells and DCs within SLOs. Thus, lack of CCR7 results in delayed activation of the adaptive immune system. To investigate the importance of CCR7 for atherogenesis, we crossed ccr7−/− mice on an atherosclerosis-prone ldlr−/− background. Here, we report that atherosclerotic plaque extent throughout the aorta was profoundly reduced in ccr7−/−/ldlr−/− mice after 12 weeks of a high-cholesterol diet. Additionally, we could demonstrate a decreased proportion of macrophages in atherosclerotic plaques of ccr7−/−/ldlr−/− mice (CCR7−/−) injected with T cells cocultivated with oxLDL- or nLDL-pulsed DCs. Mice injected with ccr7−/−/ldlr−/− T cells pulsed with oxLDL show significantly fewer macrophages within the atherosclerotic lesion than mice injected with WT T cells pulsed with oxLDL (n=5 animals per group; 3 to 5 sections per animal; mean±SEM; 1-way ANOVA followed by the Holm-Sidak method, *P<0.05 vs ldlr−/−/ mice).

Figure 6. Analysis of CCR7−/− and ccr7−/−/ldlr−/− mice injected with WT T cells (CCR7+/+) or ccr7−/−-derived T cells cocultivated with nLDL- or oxLDL-stimulated DCs during a cholesteral-rich diet. A. En face preparation of aortas (left; bar=1 cm) and aortic roots (right panel; ×100 magnification) stained with Oil Red O demonstrated that WT T cells cocultivated on oxLDL-pulsed DCs, when injected into ccr7−/−/ mice (CCR7−/−), on a high-cholesterol diet, established an atherosclerotic phenotype comparable to ldlr−/−/ mice (CCR7+/+), whereas ccr7−/−-derived T cells failed to establish atherosclerosis when injected into ccr7−/−/ldlr−/− mice. T cells cocultivated on nLDL-pulsed DCs had no influence on plaque development. Red indicates lipid-laden areas.

B. Quantification of Oil Red O-stained areas as percentage of whole aortic area (n=5 to 14 animals per group; *P<0.05 vs ldlr−/−/ mice) or as a total lesion area (mm²) in the aortic root (n=5 to 14 animals per group; 3 to 5 sections per animal; **P<0.05 vs ldlr−/−/ mice). Horizontal bars represent median values compared by Kruskal-Wallis test followed by the Dunn posthoc test. C. Representative immunohistochemical staining of macrophages (MOMA-2) in aortic plaques of ccr7−/−/ldlr−/− mice after injection of WT- and ccr7−/−-derived T cells primed ex vivo with oxLDL- or nLDL-pulsed DCs (×100 magnification). D. Quantification of MOMA-2 positively stained area as percentage of total plaque area revealed more macrophages within the atherosclerotic lesions of ccr7−/−/ldlr−/− mice (CCR7−/−) injected with T cells cocultivated with oxLDL-pulsed DCs than with nLDL-pulsed DCs. Mice injected with ccr7−/−/ldlr−/−-derived T cells pulsed with oxLDL showed significantly fewer macrophages within the atherosclerotic lesion than mice injected with WT T cells pulsed with oxLDL (n=5 animals per group; 3 to 5 sections per animal; mean±SEM; 1-way ANOVA followed by the Holm-Sidak method, *P<0.05 vs ldlr−/−/ mice).
CD11c<sup>+</sup> cells in SLOs was markedly reduced. They attribute this finding to an impaired migration of CCR7-deficient CD11c<sup>+</sup> cells to SLOs to present antigens and to evoke an immune response. However, a clear discrimination between DCs and macrophages is hardly feasible because both cell types express CD11c. In addition, we found an increase in CD3<sup>+</sup> and CD4<sup>+</sup> T cells, particularly in the adventitia of atherosclerotic plaques of ccr7<sup>−/−</sup>/ldlr<sup>−/−</sup> mice. This observation is consistent with results from other groups demonstrating that ccr7<sup>−/−</sup> T cells accumulate in inflamed tissue, whereas ccr7<sup>+/+</sup> T cells emigrate from these tissues via the afferent lymph, which is said to have important implications for the generation and maintenance of the immune response.

Both T-cell activation and differentiation have been associated with atherogenesis. In particular, Th1 cells, characterized by secretion of INFγ, tumor necrosis factor-α, and interleukin-2, have been implicated to be proatherogenic. For example, Th1 cytokines trigger macrophages to release chemokine (C-C motif) ligand 2, thereby mediating the recruitment of monocytes and activated T cells to atherosclerotic lesions and promoting further disease progression. In the present study, we found reduced systemic INFγ levels, indicating a decrease in proinflammatory Th1 cell differentiation. In addition, we measured the expression of T-cell receptor Vβ6, which has been demonstrated to be the predominant T-cell receptor in atherosclerotic lesions and linked to disease activity. Accordingly, Vβ6 expression was significantly reduced in the aorta of ccr7<sup>−/−</sup>/ldlr<sup>−/−</sup> mice, pointing to an impaired activation of ccr7<sup>−/−</sup> T cells within the aorta.

To approach the question of whether loss of CCR7 in T cells or in antigen-presenting cells is more important in this setting, we performed migration and ex vivo priming experiments with CCR7-deficient T cells. First, we investigated the migratory behavior of dye-labeled ccr7<sup>−/−</sup> and ccr7<sup>+/+</sup> T cells on injection into ldlr<sup>−/−</sup> mice while fed a cholesterol-rich diet. We observed a reduced migration of ccr7<sup>−/−</sup> T cells into the inflamed aorta compared with ccr7<sup>+/+</sup> T cells. These results indicate that both exit and initial entry of T cells into the vascular wall during atherogenesis require CCR7. Interestingly, the CCR7 ligands CCL19 and CCL21 are expressed not only within SLOs but also on endothelial cells in chronically inflamed organs. Furthermore, CCL19 and CCL21 expression is enhanced within atherosclerotic lesions of apolipoprotein E-deficient mice and in human atherosclerotic carotid plaques. Although delayed entry of ccr7<sup>−/−</sup> T cells to peripheral sites of antigen exposure may contribute to reduced atherosclerotic plaque development, we speculated that injection of ex vivo primed T cells may restore atherogenesis by compensating the loss of CCR7-coordinated contact between antigen-presenting DCs and naïve T cells in SLOs. Given the autoantigenic potential and critical importance of oxLDL in atherogenesis, we used this molecule for ex vivo maturation and activation of DCs. In line with other groups, we observed maturation and activation of DCs in response to contact with oxLDL and clonal expansion and proliferation of T cells cocultivated with oxLDL-pulsed DCs. We could also confirm an oxLDL-specific induction of TCR Vβ6. However, although injection of oxLDL-primed WT T cells into ccr7<sup>−/−</sup>/ldlr<sup>−/−</sup> mice induced the development of atherosclerotic plaques to an extent comparable to ldlr<sup>−/−</sup> mice, ccr7<sup>−/−</sup> T cells did not restore atherogenesis. Thus, T-cell priming in SLOs is important but not sufficient to promote atherosclerotic plaque development.

Taken together, CCR7-dependent trafficking of T cells such as egress from inflamed tissue into the draining lymph node and (re)entry to sites of inflammation seems to be essential for the generation and maintenance of the immune response in atherosclerosis. In contrast to a traditional paradigm, local priming processes seem to be unable to generate or maintain an adaptive immune response to promote atherosclerotic plaque development. This finding broadens our understanding of the immunoinflammatory pathophysiology of atherosclerosis and may guide the understanding to novel therapeutic strategies.

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Disclosures

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References

CLINICAL PERSPECTIVE

Modulation of both innate and adaptive immune responses has become an attractive and, in some clinical settings, successful tool to treat a variety of diseases (eg, rheumatic arthritis and different forms of cancer). The perception that the interaction between innate and adaptive immunity is also important for the development of atherosclerosis has already resulted in some attempts to influence atherosclerotic plaque development by immunomodulation. Our study strengthens the importance of secondary lymphoid organs and the impact of T-cell recirculation for the transition from innate to adaptive immune system activation during atherogenesis. Together with the fact that our findings, together with others, underline the antigenic potential of oxidized low-density lipoprotein, the study presented may lead the way to novel therapeutic strategies to fight the detrimental clinical end points of atherosclerosis. For instance, blockage of costimulatory molecules on oxidized low-density lipoprotein–pulsed dendritic cells ex vivo might positively influence atherosclerotic plaque development. In addition, artificial modulation of oxidized low-density lipoprotein presentation or interaction of dendritic cells with T cells in secondary lymphoid organs may be new ways to attenuate atherogenesis.
Chemokine Receptor 7 Knockout Attenuates Atherosclerotic Plaque Development
Maren Luchtefeld, Christina Grothusen, Andreas Gagalick, Kumaravelu Jagavelu, Harald Schuett, Uwe J.F. Tietge, Oliver Pabst, Karsten Grote, Helmut Drexler, Reinhold Förster and Bernhard Schieffer

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Supplemental Material

Expanded Methods and Results

All chemicals were obtained from Sigma (Taufkirchen, Germany) unless otherwise specified. All cell culture plates were from TTP (Trasadingen, Switzerland). Mouse CCL19 was obtained from Natutec (Frankfurt, Germany). Antibodies used in this study included rat polyclonal T-cell receptor (TCR) Vβ6, biotin conjugated (BD Biosciences, San Jose, CA), hamster polyclonal CD11c, APC conjugated (BD Pharmingen, Heidelberg, Germany), rat CD3, FITC conjugated (BD Pharmingen, Heidelberg, Germany), rat monoclonal CCR7, APC conjugated (eBioscience, San Diego), rat polyclonal MHC class II, FITC conjugated, rat polyclonal CD45, peridinin chlorophyll protein (PerCP) conjugated (BD Pharmingen, Heidelberg, Germany), goat F(ab’)2 IgG FITC conjugated (Serotec, Duesseldorf, Germany), rabbit IgG, FITC conjugated (Jackson ImmunoResearch, Suffolk, UK). Streptavidin-Fluorescein (Santa Cruz biotechnology, LA, USA), hamster monoclonal IgG FITC conjugated (Serotec, Duesseldorf, Germany), rat polyclonal MOMA-2 (Acris, Herford, Germany), rat polyclonal CD3 (Abcam, Cambridge, UK), rat polyclonal CD4 (BD Pharmingen, Heidelberg, Germany), biotin conjugated secondary antibody, goat anti-rabbit and rabbit anti-rat (Vector Laboratories, CA, USA) and custom-made mouse IgG2a-PE conjugated.

Genotyping

The genotype of each mouse was verified by PCR on genomic DNA (tail tip digest) using the following primers: LDLR sense: 5’-ACC CCA AGA CGT GCT CCC AGG ATG A-3’, LDLR antisense 5’-CGC AGT GCT CCT CAT CTG ACT TGT-3’; CCR7-Wildtyp sense: 5’-CGT GTC CTC GCC GCT GTT-3’, CCR7-Wildtyp antisense 5’-CCC CGG GCA ATG TCC TGA-3’; CCR7-knockout sense: 5’-GTC TCC GCC TCC ATG CTT CAC C-3’; CCR7-knockout antisense: 5’-CTC TCG TGG GAT CAT TGT TTT TCT-3’.
Tissue preparation

For the analysis of atherosclerotic lesion areas, aortas were prepared *en face* as described previously. Within the aortic root, lesion areas were analyzed in cross-sections obtained at the level of all 3 leaflets of the aortic valve. To assess cellular morphology, cross sections were stained with hematoxylin and eosin. The lesion area in the aortic root was determined via computer-assisted image quantification (Axio Vision, Zeiss, Jena, Germany).

For immunohistochemistry, the aortic root was isolated, embedded in OCT and cut in 6 µm cross sections. Monocytes/macrophages were detected with rat anti-mouse MOMA-2 antibody, T-cells were stained with rabbit anti-mouse CD3, rat anti-mouse CD4 antibody or respective non immune IgG subclass. Dendritic cells (DCs) were detected with CD11c antibodies. The biotinylated secondary antibody (goat anti-rabbit, rabbit anti-rat) was visualized by ABC reagent (Vector Laboratories, CA) and the ACE-Chromogen (DAKO, Glostrup, Denmark) according to the manufacturer’s protocol. Sections were counterstained with hematoxylin (Roth, Karlsruhe, Germany). Morphometric data were obtained by image analysis. While the number of CD3- and CD4+ cells was counted directly, MOMA-2 and CD11c positive areas were obtained by image analysis. (QWin software; Leica; Axiovert 200M; Carl Zeiss MicroImaging, Inc., Jena, Germany). Immunofluorescence was performed according to standard protocols. Briefly, sections were rehydrated in TBST (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.1% Tween-20), pre-incubated with TBST containing 5% rat or mouse serum, blocked with 0.001% avidin/PBS and 0.001% biotin/PBS and stained with a cocktail of biotinylated or fluorescent dye-coupled antibodies in 2.5% serum/TBST. Biotinylated antibodies were visualized by fluorescent streptavidin conjugates. Sections were mounted with MOVIOL. Composite images were automatically assembled using a motorized Axiovert 200M (Carl Zeiss MicroImaging, Inc., Jena, Germany). Immunohistological analyses of spleen and MLNs were performed as previously described using a motorized Axiovert M200.
microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). Cryosections of MLNs were blocked with rat serum and stained with antibodies against the indicated markers. Overviews of MLNs sections were achieved using automated image assembly applying the KS300 MosaiX software (Carl Zeiss MicroImaging, Inc., Jena, Germany).

**Plasma analyses**

Plasma samples were collected after an overnight fast. Total cholesterol and triglycerides were determined by colorimetric assays (WAKO Chemicals, Neuss, Germany) following separation of lipoprotein subfractions by ultracentrifugation,\textsuperscript{4} INF\textgreek{g} (eBioscience, San Diego), IL-4 (Biocat, Heidelberg, Germany), and IL-17 (R&D-Systems, Minneapolis) were measured by ELISA.

**Flow cytometry analysis of immune cells within mouse aorta and MLN**

Whole aortas were harvested and digested with collagenase type II (120 U/ml; Biochrom AG, Berlin, Germany) and elastase (0.5 mg/ml, Sigma Aldrich-Deisendorf, Germany) in DMEM at 37°C for 1 h. A cell suspension was obtained by meshing the aorta through a 70 µm cell strainer. MLNs were passed through a 70 µm cell strainer, erythrocytes were lysed by incubation with NH\textsubscript{4}CL (0.155 M) for 10 min at room temperature (RT). Cells were resuspended in 2% FCS/PBS and incubated with primary antibodies for 20 min at 4°C, washed twice with PBS and if required, incubated with secondary antibodies for additional 20 min. After washing, intensity of immunofluorescence was detected by flow cytometry (FACS Calibur flow cytometer, BD Bioscience, San Jose, CA). Data were analyzed by cell quest pro software (BD Bioscience, San Jose, CA). In some experiments, the aortas from two or three mice were pooled and analyzed.

**LDL-isolation**
For in vitro-experiments, LDL was isolated from human plasma by sequential gradient ultracentrifugation. The LDL cholesterol fraction was dialyzed at 4°C against phosphate buffer (140 mmol/L NaCl, 1.9 mmol/L NaH₂HPO₄, 8.1 mmol/L Na₂HPO₄). Protein concentration was determined by the Bradford method. nLDL was stored in the dark at 4°C up to six days prior use.

**LDL-peroxidation in vitro**

nLDL (100 µg/mL) was incubated with CuSO₄ (10 µmol/L) in cell culture medium for 24 hours. LDL-peroxidation was stopped with EDTA (5 mmol/L) and butylhydroxytoluol (20 µmol/L). LDL-peroxidation in vitro was analyzed by detection of conjugated diene formation by measuring UV absorbance at 234 nm. Additionally malondialdehyde as the lipid peroxidation product was measured using the thiobarbituric acid-reactive substances (TBARS assay kit, Oxitech, Buffalo, NY).

**Cell culture**

To generate DCs, bone marrow cells (BMC) derived from C57Bl/6 or CCR7-deficient mice were isolated and cultivated in RPMI + 10 % FCS+ 1% PS, 50 M β-mercaptoethanol and GM-CSF (100 ng/mL) for 6 days. The majority of these cells was positive for CD11c (>90 %). Prior stimulation with nLDL (10 or 50 µg/mL), oxLDL (10 or 50 µg/mL), and LPS (50 ng/mL) for 24 hours, cells were washed and serum-starved for two hours. T-cells were obtained by passing spleens from C57Bl/6 WT-mice (6-8 weeks) through a stainless mesh, following negative selection using the Pan T-cell Isolation Kit (Miltenyi Biotech, Gladbach, Germany). The majority of these isolated cells was positive for the common T-cell antigen CD3 (>90%).

**Real-time PCR**
Real-time PCRs were performed in triplicates in a total volume of 25 µl using brilliant SYBR green PCR master mixture (Stratagene, La Jolla, CA) on a 7300 Real-Time-PCR System (Applied Biosystems, Foster city, CA) in 96-well PCR plates (Applied Biosystems, Foster city, CA). Real-time PCR was done with an initial denaturation step at 95°C for 10 min followed by 40 PCR cycles, each cycle consisting of 95°C for 15 sec, 60°C for 1 min, and 72°C for 1 min, and SYBR Green fluorescence emission was monitored after each cycle. For normalization, expression of CD3 was determined in parallel in triplicate as an endogenous control. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences for each gene are given in the Online Data Supplement (supplemental table 1).

Migration experiment

$5 \times 10^5$ serum-starved DCs from C57Bl/6 or CCR7-KO mice were used per transwell (Costar; Corning Life Sciences, Acton, MA) to measure migration through 5.0 µm pore size membranes in response to murine CCL19 (100 ng/mL) after stimulation with nLDL (50 µg/mL) oxLDL (50 µg/mL) or LPS (50 ng/mL) for 24 hours. Cells were incubated in a humidified atmosphere of 5% CO$_2$ in air for 2 hours at 37°C. Transwells were then removed and migrated DCs counted. Each assay was performed in duplicate, and an average value was used to determine the chemotactic index (ratio of the number of cells migrated in response to chemokine compared to migration to medium alone). Migration experiments were performed at least in four independent experiments.

Flow cytometry analysis of DCs and T-cells

DCs and T-cells were incubated with primary antibodies for 20 min at 4°C, washed twice with PBS and if required, incubated with secondary antibodies for additional 20 min. After washing, immunofluorescence was detected by flow cytometry (FACS Calibur flow cytometer; BD Bioscience, San Jose, CA).
Antigen presentation assay

In vitro antigen presentations were conducted in 10 cm² cell culture plates for rescue experiments, in 6-well plates for FACS-analysis and the identification of oxLDL specific T-cell receptor chains, and in 96-well flat-bottom culture plates for proliferation assays. For proliferations assays, DCs were irradiated with 15 Gy to eliminate their proliferative capacity prior to stimulation. DCs were stimulated with nLDL (10 and 50 µg/mL), oxLDL (10 and 50 µg/mL) or LPS (50 ng/mL) for 24 hours, extensively washed and co-cultivated with purified splenic T-cells (96-well plate: 2 x 10⁵ T-cells/well, 6-well plate: 5 x 10⁵ T-cells/well, 10 cm² dish: 6 x 10⁶ T-cells/dish, DC-T-cell ratio 2:1) for three days. After three days, T-cells were collected and seeded on stimulated DCs for another 3 days. After 3 days DCs were harvested, and T-cells were again collected and seeded on stimulated DCs for one (proliferation assay) or three days. All in all, T-cells were co-cultivated for 7 or 9 days.

Rescue experiment

At 8-10 weeks of age, male $ccr7^{-/-}/ldlr^{-/-}$-mice were randomly divided into four groups (n=5 per group): (1) transfer of purified splenic CD3⁺-cells co-cultivated with oxLDL stimulated DCs, (2) transfer of purified splenic CD3⁺-cells co-cultivated with nLDL stimulated DCs, (3) PBS injection, (4) transfer of purified splenic CCR7⁻⁻-derived CD3⁺-cells co-cultivated with oxLDL stimulated DCs. $Ccr7^{+/+}/ldlr^{-/-}$-mice were used as positive controls. Group 1 and 2 received 1x10⁷ wild type derived-CD3⁺ T-cells, group 4 recieved 1x10⁷ CCR7⁻⁻-derived CD3⁺ T-cells intravenously ⁸, ⁹, every three weeks for 3 months of high-cholesterol dieting. Afterwards, atherosclerotic plaque formation was analyzed by en face preparation and oil red staining of the aorta and the aortic root.

Proliferation assay
Following co-cultivation of T-cells with stimulated DCs, T-cell proliferation was measured on the basis of DNA synthesis by 5-bromo-2′-deoxyuridine (BrdU) incorporation with a commercial colorimetric quantification kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. The amount of reaction product was determined by measuring the absorbance at 450 nm using a plate reader 6 (μQuant; Bio-Tek Instruments, Bad Friedrichshall, Germany).

**In vivo trafficking experiments**

Competitive homing assays were conducted by staining CCR7−/− and C57BL/6 splenic CD3+ with either 5 µM cell tracker green or cell tracker orange (Invitrogen) in DMEM at 37°C for 45 min and washed twice in PBS containing 1% FCS. Labelled cells were mixed at a 1:1 ratio in the starting population, and 0.5 x 10⁷ labelled cells of each population were injected i.v. into recipient mice. At 48 h, aortas, spleen, blood, and MLN were harvested 10. Aortas were digested as described in flow cytometry analysis and cell suspensions from aortas, spleen, and blood were stained with Ab against CD45. The percentages CCR7−/− T-cells and wild type T cells in the total population of CD45+ cells were determined by flow cytometry.
Supplemental Table 1: Primer sequence for Real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ – 3’</th>
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<tr>
<td>β-actin sense</td>
<td>CAT GTA TGT TGC TAT CCA GGC</td>
</tr>
<tr>
<td>β-actin antisense</td>
<td>CTC CTT AAT GTC ACG CAC GAT</td>
</tr>
<tr>
<td>CCR7 sense</td>
<td>ACA GCC CCC AGA GCA CC</td>
</tr>
<tr>
<td>CCR7 antisense</td>
<td>GAG CCA CCA CCA GCA CGT</td>
</tr>
<tr>
<td>MHC-II sense</td>
<td>CCT TCA TCC CTT CTG ACG AT</td>
</tr>
<tr>
<td>MHC-II antisense</td>
<td>TCT TCC CTG AAG AGG GAC AC</td>
</tr>
</tbody>
</table>
**Supplemental Figure 1. Impact of oxLDL-loading of dendritic cells cell migration.**

Dendritic cells (DCs) remained unstimulated (control) or were stimulated with nLDL, oxLDL (10 and 50 µg/ml) or LPS (50 ng/mL) for 24 hours. A, Enhanced migration of DCs towards CCL19 after preincubation with oxLDL compared to nLDL (n=5 independent experiments; median with 25th and 75th percentiles; Kruskal-Wallis test followed by the Dunn post hoc test; *P<0.05 vs. control). B, ccr7^{-/-}-derived DCs failed to migrate towards CCL19 after preincubation with oxLDL (n=5 independent experiments, median with 25th and 75th percentiles; Kruskal-Wallis test followed by the Dunn post hoc test; *P<0.05 vs. control).
Supplemental Figure 2. Migratory path of T-cells in atherosclerosis.

1. DC precursors are recruited from the blood to the atherosclerotic lesions, where oxLDL-as the major antigen in atherosclerosis- is captured and processed. Antigen-uptake induces DC maturation, which is associated with the up-regulation of CCR7. 2. Antigen activated DCs and naive T-cells migrate CCR7-dependent into the SLOs, where MHC-II restricted antigen presentation by DCs induces T-cell activation. 3. Following activation, T-cells migrate into the inflamed aorta and re-enter the draining lymph node which seems to be essential to generate and maintain the immune response in atherosclerosis.
Supplemental References


