Differential Influence of Chemokine Receptors CCR2 and CXCR3 in Development of Atherosclerosis In Vivo

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Background—Recruitment of mononuclear leukocytes within atherosclerotic lesions is a critical step in atherogenesis. Mice lacking the chemokine receptor CCR2, highly expressed on macrophages but also on T lymphocytes, show a striking reduction of atherosclerotic lesion formation. The chemokine receptor CXCR3 is a marker of activated T helper type 1 lymphocytes, the principal T lymphocyte type detected within atheroma. We investigated whether the deletion of both of these 2 important receptors expressed on the principal inflammatory cells present in atheroma would further affect atherogenesis in vivo.

Methods and Results—We crossed ApoE−/− mice with either CCR2−/− or CXCR3−/− mice and crossed ApoE−/− CCR2−/− mice with the ApoE−/− CXCR3−/− mice to generate a triple knockout strain. Analysis of atherosclerosis development after 10 weeks of high-cholesterol diet revealed differential effects on early atherosclerotic lesions in the abdominal aorta and on advanced lesions in aortic roots. ApoE−/− CXCR3−/− mice, but not the triple knockout mice, displayed significantly reduced atherosclerotic lesion development within abdominal aortas compared with ApoE−/− CCR2−/− and ApoE−/− mice. This reduction of lesion formation correlated with an upregulation of ant-inflammatory molecules such as interleukin-10, interleukin-18BP, and endothelial nitric oxide synthase and with an increased number of regulatory T lymphocytes within atherosclerotic lesions. In contrast, lesion size development within the aortic roots was more enhanced in ApoE−/− and ApoE−/− CXCR3−/− mice compared with ApoE−/− CCR2−/− and triple knockout mice.

Conclusions—Blocking chemokine signaling in vivo through deletion of the chemokine receptors CCR2 and CXCR3 has differential effects during atherogenesis. In addition, our results point to an important role of regulatory T lymphocytes during early atherogenesis. (Circulation. 2005;112:870-878.)

Key Words: atherogenesis ■ chemokine ■ immunology ■ inflammation ■ leukocytes

Atherosclerosis is an inflammatory disease beginning early in childhood that may lead to severe clinical complications later in life. Atherosclerosis is a progressive multifactorial process characterized by the subendothelial intimal accumulation of lipid-rich macrophages and T lymphocytes, followed by lesions composed of layers of foam cells and proliferating smooth muscle cells (SMCs) with deposition of extracellular matrix.1,2,3 Endothelial dysfunction caused by several cardiovascular risk factors, including features related to lifestyle such as smoking and obesity as well as intrinsic factors such as hypertension, hypercholesterolemia, and diabetes, triggers the migration of monocyte/macrophages and T lymphocytes toward the intima of the vessel wall. Proinflammatory factors, such as cytokines and chemokines, released from endothelial cells, macrophages, or T lymphocytes induce proliferation and migration of SMCs, as well as the recruitment of additional immunoinflammatory cells, resulting in further growth of the early atherosclerotic lesion. The chemokine receptor CCR2 is highly expressed on macrophages, and deficiency of this receptor results in a reduction of advanced atherosclerotic lesion formation in mice.4 Furthermore, it has been demonstrated that the principal T cell type detected within atheroma, T helper type 1 (Th1) lymphocytes, express high levels of the chemokine receptor CXCR3.5 In addition, it has been shown that blockade of CXCR3 results in diminished recruitment of Th1 cells at sites of inflammation.6 Recent articles have suggested an important role for T lymphocytes during early atherogenesis.7 Indeed, lymphocyte-deficient RAG1-null mice have reduced atherosclerotic lesion development after 8 weeks of diet, but not at later time points.8 Furthermore, it has been shown that accumulation of lymphocytes within lesions of murine aortas displayed an inverse association with the degree of atherosclerosis.9

To demonstrate whether the deletion of 2 main chemokine receptors expressed on the principal inflammatory cell types...
present within atheroma has additional effects on atherogenesis in vivo, we generated double ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> and ApoE<sup>-/-</sup> CXCR3<sup>-/-</sup> knockout (KO) mice and ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> CXCR3<sup>-/-</sup> triple KO mice. In the present report we describe that the chemokine receptors CCR2 and CXCR3 have differential effects on the development of atherosclerotic lesions. In addition, our results indicate that T lymphocytes play a short but important role during early atherogenesis.

**Methods**

**Animals and Tissue Processing**

Both CCR2<sup>-/-</sup> and CXCR3<sup>-/-</sup> mice had a C57BL/6J background and were generated as previously described. Each strain was backcrossed to C57BL/6J background at least 10 times. We crossed ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> mice with the ApoE<sup>-/-</sup> CXCR3<sup>-/-</sup> mice to generate a triple KO strain. CXCR3<sup>-/-</sup> mice were genotyped by polymerase chain reaction (PCR). The PCR consisted of an initial step at 94°C for 5 minutes, followed by 30 cycles: 94°C for 1 minute, 65°C for 30 seconds, and 72°C for 2 minutes. Addition of an elongation cycle at 72°C for 5 minutes ended the reaction. Forward and reverse primer sequences and product size were as follows: CXCR3 WT: 5′-GCACGCCACCCATGTCAGC-3′, 5′-AGATGAGCCTGCCGGTGGGG-3′ (589 bp); CXCR3 KO: 5′-TTCTCTGACTCCCGGCCTGGC-3′, 5′-CATCGACTGTGGCCGGCTGGG (976 bp). Genotyping for ApoE<sup>-/-</sup> and CCR2<sup>-/-</sup> was performed as previously described. As a model of in vivo atherosclerosis, 8-week-old littermate male ApoE<sup>-/-</sup>, ApoE<sup>-/-</sup> CCR2<sup>-/-</sup>, ApoE<sup>-/-</sup> CXCR3<sup>-/-</sup>, or ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> CXCR3<sup>-/-</sup> C57BL/6J mice were fed with a high-cholesterol diet (1.25% cholesterol, 0% cholate; product No. D12108, Research Diets) and kept in conventional housing. After 10 weeks of diet, mice (n=8 per group for histological analysis and n=8 per group for mRNA analysis) were killed, and vascular lesions in the aorta were analyzed. Presence of macrophages, T lymphocytes, and SMCs within lesions was determined by immunohistochemistry with rat anti-mouse CD3 (Pharmingen, clone 17A2), rabbit anti-mouse CD4 (Pharmingen, clone H129.19), rat anti-mouse Mac-3 (Pharmingen, clone M3/84), and rabbit anti-mouse smooth muscle myosin (Biomedical Technologies, clone BT-562). Percentages of positive areas were reported as areas within intima as previously described.

**Atherosclerotic Lesion Size Quantification Method**

The extent of atherosclerosis was assessed on the aortic roots and the thoracoabdominal aorta by staining for lipid deposition with Sudan IV. Atherosclerotic lesions, stained with Sudan IV for lipid deposition, were examined on thoracoabdominal aortas (Figure 1C). Atherosclerotic plague development was assessed on the aortic roots. In addition, our results indicate that T lymphocytes play a short but important role during early atherogenesis.

**Analysis of Gene Expression by Real-Time Quantitative Reverse Transcription–PCR**

Total murine mRNA was extracted from the aorta (from the beginning of the aortic arch, just after the aortic roots, to the iliac bifurcation) and prepared with TRI Reagent (MRC Inc) according to the manufacturer’s instructions. Real-time quantitative reverse transcription (RT)–PCR (ABI Prism 7000 Sequence Detection System, Applied Biosystems) was used to determine the mRNA levels of CCR1, CCR5, interferon (IFN)-γ, and interleukin (IL)-10 (ABI Prism, Pre-Developed TaqMan Assay Reagents; 6-carboxyfluorescein labeled). CD4, CD25, GATA-3, Foxp3, TIM-3, endothelial nitric oxide synthase (eNOS), and IL-18BP primers and probes were designed as described with the use of Primer Express software (Perkin-Elmer). GATA-3 primers and probe were as follows: S′-CAGAACCAGGGCCCTTTATA (forward); S′-CATTAGCGTTCCTCTCCAGA (reverse); S′-FAM-CGAAAGCCTCGCGCA-TAMRA. Each sample was analyzed in triplicate and normalized in multiplex reaction with the use of TaqMan eukaryotic 18S control (TaqMan Reagent, Applied Biosystems; VIC labeled). PCR products for the cellular markers CD4, CD25, Foxp3, TIM-3, and CCR4 were cloned in pGEM-T Easy Vectors (Promega) and used as standards for absolute quantification. Data (g×10<sup>-10</sup>) were calculated by comparing the threshold cycle value with that of the cDNA standard curve. The fold inductions of mRNA levels for CCR1, CCR5, IFN-γ, IL-10, IL-18BP, and eNOS were analyzed by the comparative C<sub>T</sub> method.

**Regulatory T Lymphocyte Isolation and Transfer**

Leukocytes isolated from the spleen and lymph nodes of male ApoE<sup>-/-</sup> mice were separated with the CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s guidelines. To assess the purity of the isolation, cells were additionally stained with CD4-FITC (Pharmering) and analyzed by FACS. Regulatory T lymphocytes were stained with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE: 1 μmol/L) for 10 minutes at 37°C. After 3 washing steps, 2×10<sup>6</sup> regulatory T lymphocytes were resuspended in RPMI containing 0.5% FCS and injected intravenously via the tail vein in female ApoE<sup>-/-</sup> recipient mice fed with a high-cholesterol diet for 8 weeks before injection. Three days after injection, the recruitment of regulatory T lymphocytes was analyzed in cryostat sections (5 μm) of the aortic roots by fluorescence microscopy. Nuclei were stained with 4′,6-diamidino-2-phénylindole (DAPI). Real-time PCR was used to quantify recruited regulatory T cells in recipient aortas as previously described. Primers and probe for the sex-determining region on the Y-chromosome (SRY) were designed with the use of Primer Express software (Perkin Elmer): S′-CATGCAAAATACAGATCACAGAA (forward); 5′-CGGCTTCTGGAAAGCTTTTCCC (reverse); S′-FAM-CAGCTGGGATCGAGTAMRA (probe). Each reaction contained 25 ng genomic DNA, and a standard curve for measuring SRY was included in each run. The number of male donor regulatory cells was calculated by comparing the threshold cycle values of the test sample with that of a standard curve prepared from serial dilutions of donor cells.

**Blood Analysis and Leukocyte Quantification**

Blood samples were collected at the beginning and end of the diet. Hematocrit and leukocyte counts were measured, and sera were used to measure cholesterol and triglyceride content.

**Statistical Analysis**

All results are expressed as mean±SEM. Two-tailed Student t test was used to detect significant differences when 2 groups were compared. For analysis of lesion size, comparison between groups was performed by use of the Mann-Whitney U Wilcoxon sum test.

**Results**

**Atherosclerotic Plaque Development**

Atherosclerotic lesions, stained with Sudan IV for lipid deposition, were examined on thoracoabdominal aortas (Figure 1A) and aortic roots of mice after 10 weeks of a high-cholesterol diet (Figure 1C). ApoE<sup>-/-</sup> CXCR3<sup>-/-</sup> mice showed a significant reduction in lipid deposition within the thoracoabdominal aorta (4.5±0.5%) compared with control ApoE<sup>-/-</sup> (7.9±1.0%) and ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> mice (9.7±0.8%), as well as the triple KO mice (6.2±0.6%) compared with ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> mice (Figure 1B). As described previously, we found no difference between ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup> CCR2<sup>-/-</sup> on the thoracoabdominal aortas. In contrast, at the site of the aortic roots, ApoE<sup>-/-</sup> CXCR3<sup>-/-</sup> mice exhibited no reduction in lesion extent com-
pared with ApoE\(^{-/-}\) (4.1±0.4 versus 4.2±0.7), but triple KO mice (1.9±0.4) had significantly fewer atherosclerotic lesions than ApoE\(^{-/-}\) mice (Figure 1D). In addition, ApoE\(^{-/-}\) CCR2\(^{-/-}\) mice had a significant reduction of lesion size within aortic roots (1.6±0.3), which confirmed previous observations.\(^4\) No differences in weight, peripheral blood leukocyte count, and total cholesterol or triglyceride values between the 4 different mouse strains were observed (Table).

**Cellular Composition of Atherosclerotic Lesions**

Because atherosclerosis is described as an inflammatory disease, cellular composition of atherosclerotic lesions rather than lesion size is of crucial importance in both atherogenesis and plaque rupture. Thus, we performed quantitative immunohistochemistry analyses for macrophages (Mac-3), T lymphocytes (CD3), T helper lymphocytes (CD4), and SMCs (myosin heavy chain) and normalized the results to the aortic lesion areas. As expected, ApoE\(^{-/-}\) CCR2\(^{-/-}\) mice showed less recruitment of macrophages than control ApoE\(^{-/-}\) mice (Figure 2A), whereas in ApoE\(^{-/-}\) CXCR3\(^{-/-}\) mice, recruitment of T helper lymphocytes was inhibited (Figure 2B). In addition, triple KO animals showed less macrophage recruitment than ApoE\(^{-/-}\) mice, an effect that was absent in ApoE\(^{-/-}\) CXCR3\(^{-/-}\) mice (Figure 2A). In contrast, significantly more T helper lymphocytes were detected in ApoE\(^{-/-}\) CCR2\(^{-/-}\) mice than in ApoE\(^{-/-}\) but not in triple KO mice (Figure 2B). The investigations with CD3 showed results similar to those obtained with CD4 (data not shown). Furthermore, the amount of SMCs per lesion area was significantly increased in all chemokine receptor KO groups compared with control ApoE\(^{-/-}\) mice (Figure 2C). The observed

**Figure 1.** Differential influence of chemokine receptors CCR2 and CXCR3. Mice developed early atherosclerotic lesions on the thoracoabdominal aorta and advanced lesions on aortic roots. Atherosclerotic lesions were measured by lipid deposition detected with Sudan IV staining, represented in red, within the thoracoabdominal aortas (A) and aortic roots (C). B and D, Quantification of lipid deposition. Bar=500 μm. Values are mean±SEM; n=8 per group. *P<0.05.
increase in SMC content was more important in ApoE/−/−CCR2/−/− and triple KO than in ApoE/−/−CXCR3−/− mice.

**Messenger RNA Analysis of Proinflammatory and Antiinflammatory Molecules**

To investigate the atherosclerotic lesions in more detail, mice from the different KO strains were again fed a high-cholesterol diet for 10 weeks, and aortic mRNAs were isolated for quantitative real-time PCR. Analyses of these mRNA samples showed significant increases in the expression of antiinflammatory mediators IL-10, eNOS, and IL-18BP within ApoE/−/− CXCR3−/− mice compared with the other mouse strains (Figure 3A to 3C). Similarly, expression of the proinflammatory cytokine IFN-γ was also higher in the ApoE/−/− CXCR3−/− mice but only showed significant differences compared with the ApoE/−/− control group (Figure 3D). Expression of the cytokine IL-4 could be detected in none of the KO groups (data not shown). Expression of the chemokines RANTES, MIP-1α, and MIP-1β did not show significant variation between the various groups (data not shown). Determination of mRNA for the chemokine receptors CCR1 and CXCR5 revealed significantly higher levels of both chemokine receptors in ApoE/−/− mice compared with all the other groups, and ApoE/−/− CXCR3−/− mice also had significantly higher values than ApoE/−/− CCR2−/− and triple KO mice (Figure 4A, 4B).

**Messenger RNA Analysis of Th1 Cell Markers**

To investigate whether these different expression patterns of the proinflammatory and antiinflammatory molecules correlated with the amount of inflammatory cell types present within the atherosclerotic vascular wall, we first analyzed mRNA levels of CD4 as a marker of T helper lymphocytes. Interestingly, these results revealed significantly higher levels of CD4 mRNA within aortic tissue of ApoE/−/− CXCR3−/− compared with the other groups (Figure 5A). Similar results were obtained for CD3 (data not shown). To determine in more detail this subpopulation of T helper lymphocytes, we first analyzed the mRNA level of TIM-3 (T lymphocyte immunoglobulin domain, mucin domain) as a marker for Th1 cells. This revealed a significantly higher TIM-3 level within aortic tissues of ApoE/−/− mice compared with the other groups (Figure 5B). However, no differences were measured between ApoE/−/− CCR2−/−, ApoE/−/− CXCR3−/−, and triple KO mice. Analysis of GATA-3 expression, a Th2 cell–specific transcription factor, revealed a significant increase in the ApoE/−/− CXCR3−/− group compared with the ApoE/−/− CCR2−/− group (Figure 5C). We performed further analyses by using the marker CD25, which is expressed on activated cells and regulatory T lymphocytes. Surprisingly, ApoE/−/− CXCR3−/− mice expressed significantly higher amounts of CD25 compared with ApoE/−/− and ApoE/−/− CCR2−/− animals but not compared with the triple KO strain (Figure 5D). However, no difference was detected between the ApoE/−/−, ApoE/−/− CCR2−/−, and triple KO mice. To further investigate a possible modulation of regulatory T lymphocytes within the various KO strains, we finally used the forkhead/winged helix transcription factor (Foxp3) as a marker for regulatory T cells. As expected, ApoE/−/− CXCR3−/− mice showed significantly higher Foxp3 mRNA levels than each of the other mouse strains. Interestingly, ApoE/−/− CCR2−/− had significantly lower amounts of Foxp3 mRNA than the control ApoE/−/− mice (Figure 5E).

**Recruitment of Regulatory T Cells Into Aortic Lesions**

The recruitment of regulatory T lymphocytes within atherosclerotic lesions was investigated in more detail to further
assess their implication in the development of atherogenesis. Isolated CD4+/CD25+ regulatory T lymphocytes obtained from ApoE−/− male mice were labeled with CFSE and injected (intravenously) in recipient ApoE−/− female mice fed a high-cholesterol diet for 8 weeks. Three days after injection, atheromas of the aortic roots were analyzed by fluorescence microscopy for CFSE-positive cells. As shown in Figure 6A, injected regulatory T lymphocytes could be detected within atheromas. To quantify the amount of recruited cells, we performed real-time quantitative PCR of DNA isolated from aortas of recipient mice. Analysis of the sex-determining region on the Y-chromosome (SRY) as a marker for injected male donor cells revealed 2.27% of injected cells recruited to the aortic wall of recipient mice.

**Discussion**

On the basis of data from the literature showing the importance of chemokine and chemokine receptor interactions during atherogenesis, we decided to investigate whether the combined deletion of CCR2 and CXCR3, major chemokine receptors expressed on macrophages or T lymphocytes, respectively (the principal inflammatory cell types present within atheromas), would have additional effects on atherogenesis in vivo. Thus, we crossed ApoE-deficient mice with CCR2−/− and CXCR3−/− mice and the double KO mice together to generate a triple KO strain. As we recently described, 10 weeks of a Western-type diet induced early atherosclerotic lesions along the thoracoabdominal aorta and advanced lesions at the aortic root site in ApoE−/− mice.7 These observations confirm previous studies that demonstrated that the development of atherosclerotic lesions progresses from an initial appearance within the aortic sinus to subsequent involvement of the proximal portion of the coronary arteries, carotid arteries, and abdominal aorta.18–20

Our results demonstrate that the chemokine receptor CXCR3

### Characteristics of Mice Before and After Diet

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<td>220±41</td>
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<td>Weight, g</td>
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Mice consumed a high-cholesterol diet for 10 weeks. Values are mean±SEM. Analyses were performed on 8 mice per group.
plays an important role during the early steps of atherogenesis. Within the thoracoabdominal aorta, lipid deposition revealed decreased atherosclerotic plaque formation in ApoE/− CXCR3−/− mice compared with ApoE/− and ApoE/− CCR2−/− mice. However, no statistical differences were obtained for ApoE/− CCR2−/− and triple KO animals compared with control ApoE/− mice. These results are in agreement with Boring et al.4 who described that atherosclerotic lesions within abdominal aorta of ApoE/− CCR2−/− mice showed significant differences compared with ApoE/− mice only after 13 weeks of a cholesterol-rich diet, but not at earlier time points. Interestingly, measurements of advanced lesions on aortic roots showed different results. The ApoE/− CCR2−/− and triple KO mice displayed dramatic reduction in atherosclerotic lesion development compared with the control ApoE/− and ApoE/− CXCR3−/− strains. Our results on ApoE/− CCR2−/− mice confirm previous reports.4,21 Surprisingly, ApoE/− CXCR3−/− animals did not display any difference in atherosclerotic lesion formation compared with controls within aortic roots. These results demonstrate that the chemokine receptor CXCR3 is of minor importance for the development of advanced complicated atherosclerotic lesions. Immunohistochemistry analyses of cellular composition within advanced atherosclerotic lesions revealed that

**Figure 4.** Expression of chemokine receptors within lesions. Results of real-time quantitative RT-PCR for chemokine receptors within the aorta are shown. CCR1 (A) and CCR5 (B) mRNA levels were determined in multiplex reactions with the eukaryotic 18S control. Values are mean±SEM; n=8 per group. *P<0.05.

**Figure 5.** Expression of different T lymphocyte subtypes within lesions. Results of real-time quantitative RT-PCR for cell markers within the aorta are shown. CD4 (A), TIM-3 (B), GATA-3 (C), CD25 (D), and Foxp3 (E) TaqMan probes were used in multiplex reactions with the eukaryotic 18S control. Values are expressed as absolute cDNA quantities and are mean±SEM; n=8 per group. *P<0.05.
deletion of CCR2 or CXCR3, as well as both chemokine receptors, led to an increase in SMC content. Thus, independently of lesion size development, reduction of either macrophages or Th1 lymphocyte recruitment results in a less proinflammatory environment and in more stable lesion formation.

In an attempt to understand the mechanism involved in the reduction of atherosclerotic lesion development in the ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice, we further performed real-time RT-PCR analyses based on mRNAs extracted from aortas. Detection of antiinflammatory molecules confirmed our first data based on lipid deposition. Steady state levels of mRNA of eNOS, IL-10, and IL-18BP in ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice were higher than in the other KO strains, thus indicating protective antiinflammatory properties at the early stages of atherogenesis in ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice that might outweigh the expression of proinflammatory molecules such as IFN-γ. However, it is of note that proinflammatory functions of IFN-γ contrast with certain aspects of its biological activity: as noted by several authors, IFN-γ displays protective antiinflammatory functions as well. Expression analysis of T helper cell–specific markers revealed interesting results. ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice had a significantly higher amount of T helper lymphocytes within the abdominal aorta as detected by CD4. Knowing that most of the T helper lymphocytes present within atherosclerotic lesions are of the Th1 lymphocyte subpopulation and that blockade or deletion of the chemokine receptor CXCR3 leads to an important reduction in migration of Th1 lymphocytes at the site of inflammation,<sup>6,23</sup> we expected lower amounts of Th1 lymphocytes in mice bearing the CXCR3 deficiency. This was confirmed by the expression level of TIM-3, a specific marker of Th1 lymphocytes.<sup>15</sup> Thus, because we observed a reduction of TIM-3 expression within ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice and because IFN-γ has been reported to be secreted mostly by Th1 lymphocytes, our data seem to indicate that a different T lymphocyte type might be involved.

It has been described that severe hypercholesterolemia is associated with a switch from Th1 to Th2,<sup>24</sup> and it is well accepted that IL-10 is a Th2–related cytokine.<sup>25,26</sup> Expression of IL-10 and GATA-3 (specific Th2 marker) was increased in ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice compared with the other mouse strains. However, this amount of Th2 lymphocyte could not explain the important increase of CD4<sup>+</sup> lymphocytes detected in ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice. Interestingly, IL-10 is not exclusively related to Th2 lymphocytes, but it has also been described more recently that IL-10 and IFN-γ are also secreted by regulatory T lymphocytes.<sup>27</sup> In addition, regulatory T cells may secrete high levels of the antiinflammatory cytokine transforming growth factor-β,<sup>27</sup> which has been shown to play an important role in atherosclerosis.<sup>28,29</sup> To clarify the possible implication of regulatory T cells, we performed further analyses for CD25, an activation marker that is expressed on regulatory T lymphocytes, as well as Foxp3, a specific marker of regulatory T lymphocytes.<sup>30,31</sup>

These results indicate a higher recruitment of regulatory T lymphocytes during early steps of atherogenesis in ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice. However, among the different T lymphocyte subtypes, the proportion of regulatory T lymphocytes is lower than the other Th1 or Th2 CD4<sup>+</sup> lymphocytes.

Recently, Mallat et al<sup>32</sup> provided the first evidence for a protective role of a subset of regulatory T cells in experimental atherosclerosis. In these experiments, cell transfer of in vitro expanded ovalbumin-specific T cell clones obtained from DO11–10 BALB/c mice reduced the development of atherosclerosis in ApoE<sup>−/−</sup> mice. In our study we propose that regulatory T lymphocytes would protect transiently ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice during the early steps of atherogenesis. With regard to the other mouse strains investigated in our study, the low amount in regulatory T lymphocytes would lead to a more inflammation-prone environment. Therefore, our results suggest that early atherosclerotic lesion development might involve protective properties of regulatory T lymphocytes even at low amounts compared with Th1 effector lymphocytes (Figure 6B). The different amounts of regulatory T lymphocytes between the various KO mouse strains might be correlated with chemokine receptor expression on regulatory T lymphocytes. Indeed, Sebastiani et al<sup>33</sup> have shown that regulatory T lymphocytes express high amounts of CCR2 but low amounts of CXCR3 and that intracellular calcium mobi-
lization within regulatory T lymphocytes was highly induced under MCP-1 stimuli. Therefore, CXCR3 deletion would then have only a small effect in regulatory T lymphocyte chemotaxis, but deletion of the chemokine receptor CCR2 might be responsible for the reduced migration of regulatory T lymphocytes into atherosclerotic lesions of mice bearing the CCR2 deficiency. This reduced number of regulatory T lymphocytes would then explain the small trend of increased lesions in the ApoE<sup>−/−</sup> CCR2<sup>−/−</sup> mice compared with ApoE<sup>−/−</sup> control. Deletion of both chemokine receptors CCR2 and CXCR3 would then lead to a reduction of Th1 and regulatory T lymphocytes within the vessel wall. Thus, the lack of significant reduction of lipid deposition within the thoracoabdominal aorta of the triple KO mice could be explained by the reduced amount of regulatory T lymphocytes. Therefore, it seems that both a reduction in Th1 effector lymphocytes and an increase in antiinflammatory Th2 and regulatory T lymphocytes might be implicated in reducing early atherosclerotic plaque development. However, this protective effect of regulatory T lymphocytes seems to last only for a short period because the total number of T lymphocytes within atherosclerotic lesions reduces while the plaque grows. The reduced number of Th1 lymphocytes within the different KO groups compared with the ApoE<sup>−/−</sup> control would allow a higher recruitment of regulatory T lymphocytes, leading to a weaker imbalance between effector T lymphocytes and regulatory T lymphocytes, mainly when the chemokine receptor CXCR3 is absent.

Expression patterns of the chemokine receptors CCR1 and CCR5 within the abdominal aorta matched results obtained from lipid deposition measurements in the aortic roots, indicating enhanced inflammation in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice. The upregulation of CCR1 and CCR5, possibly from birth, might result as a compensatory regulatory effect or might indicate which chemokine receptors are present on the cells infiltrating the lesions. However, our results point out that, despite the higher amounts of antiinflammatory cytokines and increased regulatory T lymphocytes present within the abdominal aorta, ApoE<sup>−/−</sup> CXCR3<sup>−/−</sup> mice are susceptible to significant inflammation as soon as the effector T lymphocyte and regulatory T lymphocyte imbalance is altered. As a result, atherosclerotic lesion formation would then compensate for the early delay.

In conclusion, blocking chemokine signaling in vivo through deletion of the chemokine receptors CCR2 and CXCR3 has differential effects on the development of atherosclerotic lesions and indicates that T lymphocytes play a major role during early atherosclerosis. Deletion of the chemokine receptor CXCR3 reduces only the early steps of atherogenesis and does not influence advanced lesion formation. In addition, analyses of proinflammatory and antiinflammatory molecules confirm the proinflammatory role of the chemokine receptor CXCR3 and indicate that the amount of regulatory T lymphocytes is of crucial importance at the early steps of atherogenesis.

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Disclosure

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