Leukocyte-Derived Interleukin 10 Is Required for Protection Against Atherosclerosis in Low-Density Lipoprotein Receptor Knockout Mice

Stéphane Potteaux, Bruno Esposito, Olivia van Oostrom, Valérie Brun, Patrice Ardouin, Hervé Groux, Alain Tedgui, Ziad Mallat

**Background**—Atherosclerosis is an immunoinflammatory disease. Here we examined the role of leukocyte-derived interleukin 10 (IL-10) on advanced atherosclerosis development in low-density lipoprotein receptor knockout (LDLr−/−) mice.

**Methods and Results**—Bone marrow cells harvested from C57BL/6 IL-10−/− and IL-10+/+ mice were transplanted into irradiated male LDLr−/− mice. Four weeks after transplantation, mice were fed a high-fat cholate-free diet for 14 weeks. Despite no differences in weights, serum total, and HDL-cholesterol levels between the 2 groups, IL-10 deficiency in leukocytes induced a >2-fold increase in lesion development in the thoracic aorta compared with controls. We also found a significant 35% increase in aortic root lesion area of IL-10−/− mice compared with IL-10+/+ mice. Furthermore, IL-10 deficiency led to a marked increase in lymphocyte and macrophage accumulation associated with a significant reduction in collagen accumulation. Finally, transfer of IL-10−/− splenocytes to LDLr−/− mice resulted in a 3-fold increase in lesion size in the aortic sinus compared with mice transplanted with IL-10+/+ splenocytes.

**Conclusion**—IL-10 expressed by leukocytes prevents exaggerated advanced atherosclerosis development and plays a critical role in modulation of cellular and collagen plaque composition, at least in part, through a modulation of the systemic immune response. (Arterioscler Thromb Vasc Biol. 2004;24:1474-1478.)

**Key Words:** atherosclerosis ■ leukocytes ■ inflammation

As in any other inflammatory situation, atherosclerosis develops in a proinflammatory context,1-3 which is, however, counterbalanced by anti-inflammatory mechanisms.4 Among all the molecules produced to downregulate the inflammatory process, interleukin 10 (IL-10) is certainly one of the most interesting cytokines. IL-10 is a pleiotropic cytokine that is expressed in human atherosclerotic plaques and is mainly produced by macrophages, T helper (Th) type-2 and T regulatory type-1 lymphocytes.5 Several studies have pointed to the anti-inflammatory and antithrombotic potentials of IL-10, including the control of tumor necrosis factor α (TNF-α) and IL-12 production,6 and the inhibition of NFκB activation7,8 matrix metalloproteinase (MMP) synthesis,9 tissue factor10 expression, and cell death.11 Altogether, these data strongly suggested that IL-10 might play a major role in atherogenesis. In the past few years, a series of studies have directly addressed the role of endogenous IL-10 in experimental models of atherosclerosis.12-14 IL-10 deficiency in C57BL/6 mice fed an atherogenic cholate-containing diet has been shown to enhance early lesion formation.12,13 Similar results have been reported recently in apolipoprotein E (apoE)/IL-10 double-knockout (KO) mice fed a chow diet.14 Local (intraplaque) changes in immune cell accumulation and activation have been reported in these previous studies, suggesting that they may have contributed to alterations in plaque size and composition. Although these changes were associated with systemic alteration in the immune response,14,15 the direct contribution of this latter to the process of atherosclerosis remains poorly understood.

Because IL-10 is produced mainly by bone marrow cells, we examined the direct role of leukocyte-derived IL-10 on the inflammatory process and development of advanced atherosclerotic lesions in irradiated low-density lipoprotein (LDL) receptor (LDLR)-deficient mice transplanted with bone marrow cells from IL-10+/+ or IL-10−/− mice. We have also examined the effects of splenocyte transfer from the reconstituted mice on the atherosclerotic process of nonirradiated LDLr−/− recipient mice.

**Methods**

**Generation of Chimeric Mice**

Fourteen-week-old C57BL/6 LDLr−/− female mice were subjected to medullar aplasia by 9.5 gray lethal total body irradiation. The mice were then transplanted with bone marrow cells from IL-10−/− or IL-10+/+ mice. The recipients were fed a 3-fold increase in lesion development in the thoracic aorta compared with controls. We also found a significant 35% increase in aortic root lesion area of IL-10−/− mice compared with IL-10+/+ mice. Furthermore, IL-10 deficiency led to a marked increase in lymphocyte and macrophage accumulation associated with a significant reduction in collagen accumulation. Finally, transfer of IL-10−/− splenocytes to LDLr−/− mice resulted in a 3-fold increase in lesion size in the aortic sinus compared with mice transplanted with IL-10+/+ splenocytes.

**Conclusion**—IL-10 expressed by leukocytes prevents exaggerated advanced atherosclerosis development and plays a critical role in modulation of cellular and collagen plaque composition, at least in part, through a modulation of the systemic immune response. (Arterioscler Thromb Vasc Biol. 2004;24:1474-1478.)

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were repopulated with an intravenous injection of bone marrow cells (2 million) isolated from femurs and tibias of 12-week-old C57BL/6 wild-type or C57BL/6 IL-10−/− mice (Charles River, Orléans, France). After 4 weeks of recovery, mice were fed a proatherogenic diet containing 15% fat, 1.25% cholesterol, and 0% cholate for 14 weeks.

### Extent and Composition of Atherosclerotic Lesions in Aortas and Aortic Sinus

Mice were anesthetized with isoflurane before being euthanized by cervical fracture. Plasma cholesterol and high-density lipoprotein were measured using a commercial cholesterol kit (Sigma). The heart and aorta of mice were removed, fixed in 4% paraformaldehyde, and placed in a PBS sucrose 30% solution overnight at 4°C, before being embedded in a cutting medium and frozen at −70°C.

Successive 10-μm transversal sections of aortic sinus were obtained. Lipids and collagen were detected using Oil red O and Sirius red stainings, respectively. The presence of macrophages, T lymphocytes, and smooth muscle cells was studied using a monoclonal rat anti-mouse macrophage antibody (clone MOMA-2, MAB1852; Chemicon), a polyclonal goat anti-CD3 antibody (Santa Cruz Biotechnology) and a monoclonal anti-α-smooth muscle actin antibody (clone 1A4; Sigma), respectively. Morphometric studies were performed in the thoracic aorta and in aortic root sections using Histolab software (Microvisions). At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used.

### Cytokine Assays

Mouse spleen-derived T cells were cultured in presence of concanavalin A (10 μg/mL) as described previously. Murine cytokine interferon-γ (IFN-γ) and IL-4 were measured in supernatants using specific ELISAs, as described previously.

### Splenocyte Transfer From Chimeric Mice

Splenocytes (25 million per mouse) from IL-10+/+ or IL-10−/− chimeric mice obtained at the time of death were injected intravenously into 6-month-old C57BL/6 LDLr KO mice kept until the time of splenocyte injection on a chow diet (n=4 injected with IL-10+/+ splenocytes; n=6 injected with IL-10−/− splenocytes). We chose 6-month-old recipient mice because these mice had an activated endothelium with already small fatty streaks of less than 10 000 μm² in the aortic sinus (data not shown). At the time of injection, mice were put on a high-fat (15% fat, 1.25% cholesterol) cholate-free diet for 6 weeks. Lesion size in the aortic sinus was assessed as indicated above.

### Statistical Analysis

Data were expressed as mean±SEM. Statistical significance was determined by use of ANOVA. P<0.05 was considered statistically significant.

### Weights, Cholesterol Levels, and Aortic Root Lesion Size and Composition

<table>
<thead>
<tr>
<th></th>
<th>IL-10+/+ (n=7)</th>
<th>IL-10−/− (n=7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weights (g)</td>
<td>25.8±1.2</td>
<td>23.8±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>10.99±1.60</td>
<td>10.33±1.21</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>0.51±0.06</td>
<td>0.52±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Lesion size in aortic sinus (mm²)</td>
<td>408±3793</td>
<td>553±175</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD3 (cells/mm²)</td>
<td>16.9±3.8</td>
<td>36.5±8.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MOMA-2 %</td>
<td>18.6±3.4</td>
<td>30.1±2.6</td>
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<tr>
<td>α-smooth muscle actin %</td>
<td>1.4±0.4</td>
<td>1.7±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Sirius red %</td>
<td>15.2±2.8</td>
<td>7.8±1.8</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

### Results

#### Effect of IL-10 Deficiency in Bone Marrow Cells on Atherosclerotic Lesion Size

Animal weights, plasma total cholesterol, and HDL-cholesterol did not differ between the 2 groups (Table). We analyzed the extent of atherosclerosis in the aortic sinus and thoracic aorta (including the brachiocephalic artery) by staining for lipids in IL-10−/− and control groups. Quantitative computer assisted-image analysis revealed more than a doubling of the extent of lipid staining in thoracic aortas of IL-10−/− mice compared with controls (16.2±2.4% versus 7.9±0.6%, respectively; P=0.006; Figure 1). We also found...
a significant 35% increase in plaque area in the aortic sinus of mice with bone marrow IL-10 deficiency compared with controls (Figure 2, Table).

Effect of IL-10 Deficiency in Bone Marrow Cells on Atherosclerotic Lesion Composition

We examined the presence of smooth muscle cells, macrophages, T lymphocytes, and the accumulation of collagen in plaques from both groups of mice. Although accumulation of α–actin–positive smooth muscle cells was similar between the 2 groups of mice (Table, Figure I, available online at http://atvb.ahajournals.org), IL-10 deficiency in bone marrow cells was associated with marked increases in both MOMA-2 and CD3 immunostainings (62% and 116%, respectively) and a significant 49% decrease in Sirius red staining (Figure 3, Table). These results reveal a greater accumulation of macrophages and lymphocytes but a lower collagen content in the plaques of mice with IL-10–deficient bone marrow compared with controls.

Effect of IL-10 Deficiency in Bone Marrow Cells on the Immune Response

The nature of the immune response is an important factor in atherosclerosis development. Therefore, we analyzed the cytokine profile of T cells from splenocytes of both groups. IL-10 deficiency was associated with a marked increase in IFN-γ production compared with the control group (2.50 ± 0.43 ng/mL and 0.81 ± 0.38 ng/mL respectively; P = 0.04). There was no difference in IL-4 production between the 2 groups. Although we did not perform a detailed analysis of the different T and B cell populations in the spleens by flow cytometry, we found no difference in spleen CD4 and CD8 immunostainings between groups (data not shown). Thus, bone marrow IL-10 deficiency in a hypercholesterolemic context resulted in a more powerful systemic Th1 response.

Effect of Splenocyte Transfer on Atherogenesis

To examine the role of the systemic Th1 response on the process of atherosclerosis, we transferred splenocytes (25 million per mouse) obtained from IL-10+/+ or IL-10−/− chimeric mice at the time of death into 6-month-old C57BL/6 LDLr KO mice. After 6 weeks on a high-fat cholate-free diet, mice injected with the IL-10−/− splenocytes showed a 3-fold increase in mean lesion area in the aortic sinus compared with mice injected with the IL-10+/+ splenocytes.
Atherosclerosis is a very common and fatal disease. Immunoinflammatory-driven processes are involved in disease evolution, from the beginning of fatty streak formation to the complications of advanced plaques.\textsuperscript{1,2,17} Recently, studies have focused on the identification of potential counter-regulatory mechanisms that could be involved in limitation of disease progression or reduction of plaque instability. As a result, anti-inflammatory and immunosuppressive cytokines such as IL-10\textsuperscript{12–15,18} and transforming growth factor-\(\beta\)\textsuperscript{19,20} have shown interesting antiatherogenic and plaque-stabilizing properties. The comprehension of these counter-regulatory mechanisms would be very useful in the elaboration of novel therapeutic strategies to act against atherosclerosis.\textsuperscript{17,21,22}

Several studies have already been conducted to determine the effect of endogenous IL-10 in atherosclerosis and demonstrated that IL-10 deficiency was able to enhance early atherosclerotic lesion formation, independent of circulating lipoprotein levels.\textsuperscript{12–14} Moreover, IL-10 supplementation or overexpression has been shown to reduce lesion size.\textsuperscript{12,15,18} An important protective role of endogenous IL-10 against development of vulnerable plaques has also been reported in apoE/IL-10 double-KO mice and was associated with an increase in MMP and tissue factor activities in IL-10–deficient plaques compared with controls.\textsuperscript{14} Although IL-10 was reported to protect against accelerated development of early lesions, the latter study found no differences in either plaque size or cellular composition in more advanced stages of the disease. However, old apoE\textsuperscript{−/−}/IL-10\textsuperscript{−/−} mice with advanced lesions presented a significant 22\% reduction in plasma total cholesterol levels, which, as we recognized, may have obscured any lesion-promoting effect of IL-10 deficiency at this advanced stage of the disease.

In the present study conducted in LDLr\textsuperscript{−/−} mice fed a high-cholesterol cholate-free diet, we show that IL-10 deficiency significantly enhances development of advanced atherosclerotic lesions in the aortic sinus in the absence of any effect on total or HDL plasma cholesterol levels. In addition, this proatherogenic potential of IL-10 deficiency was not limited to the aortic sinus because it was also observed in the thoracic aorta (including the brachiocephalic artery), where IL-10 deficiency was responsible for more than a doubling of atherosclerotic lesion size. These results obtained in mice with IL-10–deficient bone marrow cells strongly suggest that leukocytes are a major source of IL-10, required for protection against excessive growth of advanced lesions. Whether IL-10–protective effects will be sustained in very advanced stages of the disease is still unknown. However, the mean lesion size in this study compares well with those already reported in the literature.\textsuperscript{14,17,18}

To understand the mechanisms responsible for the proatherogenic effect of IL-10 deficiency, we examined the internal composition of the plaques. Although the accumulation of smooth muscle cells did not change between the 2 groups, we noticed an important increase of the proportion of macrophages and T lymphocytes and a significant reduction of collagen content in the plaques of chimeric IL-10\textsuperscript{−/−} mice in comparison with controls. This is in line with previous studies showing that IL-10 inhibits the interaction between activated endothelial cells and monocytes in an atherogenic context\textsuperscript{13} and reduces plaque MMP activity.\textsuperscript{14} Plaque inflammation and changes in collagen content might also be related to enhanced systemic Th1-mediated responses in chimeric IL-10\textsuperscript{−/−} mice, as suggested by the significant increase in IFN-\(\gamma\) production by splenocytes from chimeric IL-10\textsuperscript{−/−} mice compared with the control group. Therefore, we examined the potential role of this systemic immune response in atherosclerosis development. Our findings that the proatherogenic effects of IL-10 deficiency can be transferred by IL-10–deficient splenocytes clearly suggest an important contribution of the systemic immune response to the process of atherosclerosis. It will be important to determine in future studies the relative contribution of the various subpopulations of spleen cells (including CD4, CD8, and B cells) to this effect.

In conclusion, using a model of chimeric LDLr\textsuperscript{−/−} mice in which bone marrow cells were deficient for IL-10, we provided direct evidence that IL-10 expressed by leukocytes prevents development of advanced atherosclerotic lesions and plays a critical role in the modulation of cellular and collagen plaque composition, at least in part, through a systemic immune response modulation.

Acknowledgments

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Representative photomicrographs showing immunohistochemical staining for smooth muscle α-actin (black staining) in atherosclerotic lesions of irradiated LDLR KO mice transplanted with IL-10+/+ or IL-10−/− bone marrow and fed an atherogenic diet for 14 weeks. L indicates the lumen of the aortic sinus.