Adoptive Transfer of CD4⁺ T Cells Reactive to Modified Low-Density Lipoprotein Aggravates Atherosclerosis

Xinghua Zhou, Anna-Karin L. Robertson, Charlotta Hjerpe, Göran K. Hansson

Objective—Atherosclerosis is associated with immune responses to oxidized low-density lipoprotein (oxLDL). The presence of activated macrophages and T cells in lesions suggests that cell-mediated immune reactions are taking place during the disease process. However, the role of specific immune responses has remained unclear. We have previously shown that transfer of CD4⁺ T cells from apolipoprotein E knockout mice (apoE⁻/⁻) into immunodeficient apoE⁻/⁻ scid/scid mice accelerates disease.

Methods and Results—To test whether this effect is dependent on specific disease-associated antigens, purified CD4⁺ T cells from oxLDL-immunized mice were transferred into apoE⁻/⁻ scid/scid mice. CD4⁺ T cells from mice immunized with a nonrelevant antigen, keyhole limpet hemocyanin (KLH), and naïve CD4⁺ T cells were used as controls. After 12 weeks, all mice that received T cells had larger lesions than untouched apoE⁻/⁻ scid/scid controls. However, mice receiving CD4⁺ T cells from oxLDL immunized mice had substantially accelerated lesion progression compared with those receiving naïve or KLH-primed T cells. Circulating levels of interferon-γ were increased in proportion to the acceleration of atherosclerosis.

Conclusion—These data show that adoptive transfer of purified CD4⁺ T cells from oxLDL-immunized mice accelerates atherosclerosis. They support the notion that Th1 cellular immunity is proatherogenic and identify oxLDL as a culprit autoantigen. (Arterioscler Thromb Vasc Biol. 2006;26:864-870.)

Key Words: atherosclerosis ▪ lymphocytes ▪ low-density lipoprotein ▪ immune system ▪ mice, knockout⁻/⁻

Research over the last decades has identified immune mechanisms as pathogenically important in atherosclerosis.¹ ² T lymphocytes and macrophages are present in lesions throughout the development of disease, both in man and experimental models.³ ⁴ Most of the T cells in the lesions are CD4⁺ TCRαβ⁺ T cells expressing activation markers such as human leukocyte antigen-DR (HLA-DR) and interferon-γ (IFN-γ).⁵ ⁶ ⁷ ⁸ The expression of major histocompatibility complex (MHC) class II molecules such as HLA-DR on activated macrophages and activated T cells adjacent to these macrophages in the lesions strongly suggests that cell-mediated immune reactions are taking place in atherosclerosis.

A series of investigations have suggested modified low density lipoproteins (LDLs) as autoantigens in atherosclerosis.⁹ LDL accumulates in lesions, where it is modified by oxidative and enzymatic processes.¹⁰ ¹¹ ¹² ¹³ ¹⁴ Circulating autoantibodies to epitopes of oxidized LDL (oxLDL) have been detected in patients and experimental animals with atherosclerosis.¹⁵ ¹⁶ ¹⁷ and antibodies isolated from atherosclerotic lesions recognize oxLDL.¹⁸ Macrophages can take up, process, and present neoantigens such as oxLDL to CD4⁺ T cells, eliciting cellular immune responses.¹⁹ Indeed, ≈10% of the CD4⁺ T cells cloned from human lesions recognize oxLDL in an MHC class II–dependent manner.²⁰

The apolipoprotein E knockout (apoE⁻/⁻) mouse is a useful model of human disease because it develops severe hypercholesterolemia and spontaneous atherosclerosis.²¹ ²² In this model, a local immune response dominated by CD4⁺ T cells occurs in all phases of atherosclerosis.²³ ²⁴ T cells from immune organs of apoE⁻/⁻ mice exhibit strong reactivity toward oxLDL.²⁵ In addition, high titers of circulating autoantibodies to oxLDL are detected in this model.²⁶ However, the role of the T-cell immune response to modified LDL in the disease process has remained unclear.

To study the role of adaptive immunity in atherosclerosis, we generated an immunodeficient apoE⁻/⁻ scid/scid mouse by crossing apoE⁻/⁻ mice with the scid/scid strain, which lacks T and B cells.²⁷ We reported previously that transfer of CD4⁺ T cells from aged apoE⁻/⁻ mice into immunodeficient apoE⁻/⁻ scid/scid mice accelerates the progression of atherosclerosis.²⁸ To determine whether this effect is dependent on specific disease-associated antigens, CD4⁺ T cells from mice immunized with oxLDL were transferred into apoE⁻/⁻ scid/
scid mice. Controls included CD4+ T cells from mice immunized with a nonrelevant antigen, keyhole limpet hemocyanin (KLH), or with incomplete Freund’s adjuvant (IFA) alone, as well as untouched mice. Our results demonstrate an important role for the oxLDL-specific T-cell response as an accelerating factor in atherosclerosis.

Methods

Animals, Diets, and Serum Analysis
C57BL/6J mice obtained from Taconic Europe, Ry Denmark, and apoE−/− scid/scid mice on the C57BL/6J background were generated in our laboratory,24 kept under sterile conditions, and fed standard mouse chow. All experiments were approved by the local ethics committee. The apoE−/− scid/scid mice were injected intravenously with 50 ng of oxLDL at 7 weeks of age and euthanized at 16 weeks. To exclude a potential “leakage” (ie, appearance of immune cells), which has been reported in aged scid/scid animals, we assessed the frequency of immunocompetent cells by flow cytometry. Splenocytes were stained with fluorescein isothiocyanate–conjugated rat anti-CD3, phycoerythrin-conjugated rat anti-CD8, phycoerythrin-conjugated rat anti-CD19, and CyChrome conjugated rat anti-CD4. All antibodies were from Pharmingen. Blood was obtained by heart puncture at euthanization and allowed to clot. Sera were centrifuged at 14,000g for 20 minutes to remove chylomicrons. The serum IFN-γ, interleukin-4 (IL-4), and IL-5 levels were analyzed by sandwich ELISA with OptELA antibody sets (PharMingen) and quantitated amounts of homologous MDA-LDL or KLH for 72 hours. These cells were incubated in the absence of or in the presence of gentamicin sulfate (Sigma) after osmotic lyses of red blood cells.

Homologous LDL Isolation and Modification
Blood was obtained by heart puncture from 8- to 10-week-old apoE−/− mice and pooled into vacuum tubes containing K₂EDTA. LDL was isolated from plasma by ultracentrifugation through a discontinuous NaCl gradient of 1,020 to 1,063 mg/mL for 20 hours at 4°C in a Beckman L-80 ultracentrifuge with a 50.3-Ti Beckman fixed-angle rotor.16,25 In plasma from apoE−/− mouse, this density cutoff may contain chylomicron remnants in addition to LDL.25,26 The protein content was determined by the Lowry method, and the LDL preparation with added Na₂EDTA (1 mg/mL) was sterile filtered, kept at 4°C under N₂, and used within 2 weeks. Malondialdehyde (MDA) modification of LDL was performed as described previously.25

Immunization Protocol and Analysis of Antigen Specificity
At 6 weeks of age, male C57BL/6J donor mice were randomly divided into 3 groups (n=40 per group). Two groups were injected peritoneally with either homologous MDA-LDL (100 µg protein per mouse) or KLH (Pierce; 100 µg protein per mouse) and boosted once after 2 weeks. MDA-LDL and KLH were emulsified with IFA before immunization. The third group was used as donors of naïve T cells. Splenocytes were obtained on day 5 after the booster. The splenocytes from individual mice were cultured in duplicate in serum-free medium including 1:100 ITS + Premix (Collaborative Biomedical), 1 mg/mL BSA, 10 mmol/L HEPES, 1 mmol/L Na pyruvate, 1 mmol/L nonessential amino acids, and 50 µg/mL gentamicin sulfate (Sigma) after osmotic lyses of red blood cells. These cells were incubated in the absence of or in the presence of titrated amounts of homologous MDA-LDL or KLH for 72 hours. The incubation was followed by overnight incorporating with 3H-thymidine for the assay of cell proliferation. To quantify the T-cell cytokines secreted by the splenocytes, supernatants harvested 72 hours after incubation were measured using a Th1/Th2 Cytokine CBA Kit (BD Biosciences) according to manufacturer instruction.
Six-week-old C57BL/6J (denoted B6) rather than apoE<sup>−/−</sup> mice were chosen as donors because their T cells are largely naïve, whereas the autoimmune process starts early in apoE<sup>−/−</sup> mice. The experimental design is outlined in Figure 1. Donor B6 mice were immunized twice with MDA-LDL or the control antigen KLH. To avoid inducing a T-cell response to other antigens (eg, heat shock proteins), IFA rather than complete Freund’s adjuvant

Figure 2. Induction of antigen-specific T cells in immunized donor mice and effects of CD4<sup>+</sup> T cell transfer on circulating IFN-γ levels. A and B, Splenocyte proliferation assay. The cells from individual mouse after lyses of red blood cells were cultured in serum-free medium with titrated amounts of homologues MDA-LDL or KLH and the antigen-specific proliferation was measured by <sup>3</sup>H-thymidin incorporation. C through I, Antigen-specific T-cell cytokine secretion. Analysis of the cytokines in cultures of the cells from individual mouse was performed after 72-hour incubation with different amounts of antigens. J, IFN-γ concentration (pg/mL) in peripheral blood of the T-cell receivers at 16 weeks old. Mean±SD; n=6; *P<0.05 vs control.

CD4<sup>+</sup> T cells on atherosclerosis. Six-week-old C57BL/6J (denoted B6) rather than apoE<sup>−/−</sup> mice were chosen as donors because their T cells are largely naïve, whereas the autoimmune process starts early in apoE<sup>−/−</sup> mice.
was used in our immunization protocol. The splenocytes of MDA–LDL-immunized mice showed a dose-dependent proliferation in response to in vitro stimulation with MDA-LDL but not to KLH (Figure 2A and 2B). Strong systemic IgG responses were registered to the respective antigens (Figure I, available online at http://atvb.ahajournals.org). T-cell proliferation was accompanied by increased expression of the T-cell cytokines IFN-γ and IL-2 (Figure 2C and 2D). Splenocytes collected from KLH-primed mice exhibited significant proliferation when exposed to KLH but not to MDA-LDL in vitro and was accompanied by increased expression of the T-cell cytokines IFN-γ, IL-2, and IL-5 (Figure 2B, 2F through 2H). The results of the proliferation assay and the expression pattern of the T-cell cytokines therefore indicated the induction of antigen-specific T cells to the respective antigens used for immunization. No IL-4 production was detected in any of the groups, as is usually the case with B6 mice (data not shown). Robust IL-5 secretion was registered from the KLH-primed T cells when challenged in vitro with KLH (Figure 2I). In contrast, IL-5 level did not increase when T cells from MDA-LDL–immunized mice were exposed to their cognate antigen (ie, MDA-LDL; Figure 2I). Expression of tumor necrosis factor-α (TNF-α) increased in T cells from MDA-LDL–immunized mice in response to in vitro MDA-LDL stimulation (Figure 2E), whereas TNF-α levels were not significantly changed in KLH-primed group after stimulation with KLH (data not shown). In conclusion, a Th1 response was observed when splenocytes from mice immunized with MDA-LDL were challenged with MDA-LDL in vitro. In contrast, both Th1 and Th2 responses took place in the splenocytes primed with KLH and stimulated in vitro with KLH (Figure 2I).

**Lesion Development in ApoE<sup>−/−</sup> scid/scid Mice After Adoptive Transfer**

Compared with untreated apoE<sup>−/−</sup> scid/scid mice, fatty streak lesions in the aortic root were on average 227% larger in apoE<sup>−/−</sup> scid/scid mice that received MDA-LDL<sup>+</sup>CD4<sup>+</sup> T cells, 112% larger in the KLH<sup>+</sup>CD4<sup>+</sup> T cell transfer group, and 127% larger in the group receiving naive<sup>+</sup>CD4<sup>+</sup> T cells from untouched B6 mice (Figure 3). Lesion development was significantly increased in apoE<sup>−/−</sup> scid/scid mice that received MDA-LDL<sup>+</sup>CD4<sup>+</sup> T cells compared with those receiving either KLH<sup>+</sup>CD4<sup>+</sup> or naive<sup>+</sup>CD4<sup>+</sup> T cells (P<0.008 and 0.019; Figure 3). The latter 2 treatment groups did not differ significantly from each other with regard to lesion size but differed from lesion size in untreated mice (Figure 3).

**T-Cell Homing and Inflammatory Molecule Expression in the Lesions After Specific T-Cell Transfer**

Immunohistochemical analysis showed a strikingly increased infiltration of CD4<sup>+</sup> cells into the lesions after MDA-LDL<sup>+</sup>CD4<sup>+</sup> T cell transfer (Figure 4A and B) compared with other treatments. Furthermore, expression of the MHC class II molecule I-A<sup>+</sup> was increased several-fold in mice receiving MDA-LDL<sup>+</sup>CD4<sup>+</sup> T cells (Figure 4A and 4C). VCAM-1 expression was significantly increased in the lesions of all apoE<sup>−/−</sup> scid/scid mice that had received CD4<sup>+</sup> T cells, irrespective of the specificity of the transferred cells (Figure 4A and 4D).

### Bodyweight, Serum Lipids, and Cellular Composition in the Spleens

<table>
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<th>Phenotype of Recipient ApoE&lt;sup&gt;−/−&lt;/sup&gt; scid/scid Mice</th>
<th>Untouched</th>
<th>apoE&lt;sup&gt;−/−&lt;/sup&gt; scid/scid</th>
<th>KLH&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt; T-Cell Transfer</th>
<th>MDA-LDL&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt; T-Cell Transfer</th>
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<tr>
<td>Weight, g</td>
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<td>Serum cholesterol, mmol/L</td>
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<td>10.29±1.28</td>
<td>13.07±1.56</td>
<td>11.28±1.44</td>
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<tr>
<td>Spleen CD4&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt;, % of splenocytes</td>
<td>undetectable</td>
<td>4.66±0.47</td>
<td>6.76±1.41</td>
<td>7.10±1.64</td>
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Serum cholesterol was determined enzymatically and the cell type analyzed by flow cytometry. n=4 to 6 mice per group. Mean±SEM.

**Figure 3.** Effects of CD4<sup>+</sup> T cell transfer on atherosclerotic lesion development. Morphometric quantitation of lesion size (μm²) in aortic root from 16-week-old untreated apoE<sup>−/−</sup> scid/scid and from apoE<sup>−/−</sup> scid/scid, which received naive<sup>+</sup>CD4<sup>+</sup> T cells, KLH<sup>+</sup>CD4<sup>+</sup> T cells, or MDA-LDL<sup>+</sup>CD4<sup>+</sup> T cells. Mean±SD; *P<0.05 vs the group received CD4<sup>+</sup> T cells from untreated group; #P<0.05 vs the groups received CD4<sup>+</sup> T cells from either naïve or KLH groups.
Discussion

The results of this study show, for the first time, that adoptive transfer of cell-mediated immunity to oxLDL accelerates atherosclerosis. Thus, transfer of CD4+ T cells from MDA-LDL–immunized mice caused a 3.4-fold increase in lesion size in apoE−/− scid/scid animals. This was nearly a doubling of lesion size when compared with the situation in mice receiving CD4+ T cells from mice immunized with another antigen: KLH. This increase was accompanied by systemic elevation of the Th1 cytokine IFN-γ, by increased penetration of CD4+ T cells into lesions, and by augmented expression of (the IFN-γ–inducible) I-Ab protein in the lesions, supporting the notion that Th1 cells producing IFN-γ play an important role in the disease process. The fact that transfer of CD4 cells into immunodeficient mice aggravates the disease is in line with our previous finding that absence of CD4 cells in apoE−/− mice leads to reduced atherosclerosis.26 A proposed mechanism by which the transferred MDA-LDL–specific CD4+ T cells promote atherosclerosis was illustrated (Figure 5). It should be noted that the cholesterol levels were not different among the groups, therefore the acceleration of the disease progression was unlikely because of effects on lipid metabolism. In parallel with augmented lesion development, the mice that received MDA-LDL CD4+ T cells showed an increase in the number of CD4+ T cells in lesions. However, the proportion of CD4+ T cells in the spleen did not differ between the recipients of MDA-LDL CD4+ T cells and of KLH CD4+ T cells. These findings suggest that the MDA-LDL–specific CD4+ T cells homed to and expanded in the atherosclerotic lesions, where the MDA-LDL antigen accumulates.

A Th1 response to MDA-LDL was registered when splenocytes harvested from the mice immunized with MDA-LDL were challenged with MDA-LDL in vitro. In contrast, Binder et al reported recently that immunization with MDA-LDL induces Th2-biased responses,27 although complete Freund’s adjuvant-containing mycobacteria, which was used in their study, is well known to be more prone to induce Th1 responses than IFA, which we used.28 The discrepancy between 2 studies may be explained by the different types of adjuvant, the dose of antigen used for the immunization, the number of boost injections, the composition of the culture medium, or the concentration of antigen applied in vitro. Serum-free medium was used in our study because sera used in cell culture contain high levels of LDL, which may obscure any T-cell response to exogenous LDL particles. Importantly, the peak response of the T cells in vitro to MDA-LDL was detected when a low concentration of oxLDL was added to the cultures, whereas a concentration of oxLDL >20 μg/mL was found to induce cytotoxicity. The precise T-cell epitopes in the oxLDL preparations remain to be identified.

Interestingly, transfer of naive CD4+ or KLH CD4+ T cells resulted in modestly increased lesions with signs of increased...
It can be induced via the nuclear factorκB pathway, which is activated by proinflammatory cytokines, endotoxins, and several other stimuli. 

Among the lesions, those carrying appropriate TCR (dark gray) recognize antigen and are activated, leading to proliferation and cytokine secretion. IFN-γ, a major cytokine of activated CD4+ T cells, acts on lesion cells and initiates a cascade of inflammatory signaling that promotes disease. The CD4+ T cells that recognize nonatherosclerosis-related antigens (light gray) may act as bystanders in lesions or return to the circulation.

VCAM-1 is abundantly expressed in atherosclerotic lesions. It can be induced via the nuclear factorκB pathway, which is activated by proinflammatory cytokines, endotoxins, and several other stimuli. In the current study, VCAM-1 was induced to approx the same extent, irrespective of the antigenic specificity of transferred T cells. Our findings suggest that VCAM-1 expression reflects vascular inflammation but is less dependent on antigen specificity than I-A.

Several gene products of activated T cells are likely to be important in atherosclerosis. Numerous studies point to a proatherogenic role of Th1 products such as IFN-γ and TNF-α. Not only does IFN-γ promote antigen presentation by upregulating MHC genes, it also controls scavenger receptor expression as well as cholesterol efflux in macrophages, proliferation, differentiation and collagen production in smooth muscle cells, and hemostatic properties of the endothelium. TNF-α released by activated Th1 cells has profound effects on adhesion molecule expression, lipolytic enzymes, and several other phenomena. The cell surface molecule CD40 ligand (CD154) can activate macrophages and vascular cells, which can express CD40; this leads to secretion of proteolytic enzymes, tissue factor, and other important factors in the atherogenic process. Other costimulatory proteins expressed by immune cells include OX40L, a genotype of which was recently found to associate with coronary artery disease in man. All these effector molecules may be involved in the proatherogenic activity of CD4+ T cells, but it remains to be determined whether the properties of the activating antigens affect the effector repertoire. At any rate, our results confirm the notion that CD4+ T cells are proatherogenic; support the notion that their disease-aggravating activity may be mediated via Th1 effector molecules, and show that antigenic specificity for the disease-associated antigen oxLDL is associated with dramatic proatherogenic activity. This may be because oxLDL accumulates in the lesions, where it provides a strong local stimulus for T-cell activation.

It has been shown that systemic immunization with oxLDL decreases lesion development with significant elevation of IgG antibodies against oxLDL. The finding that immunization with MDA-LDL protects from atherosclerosis in immunocompetent apoE−/− mice whereas transfer of CD4+ T cells from mice immunized with MDA-LDL into immunodeficient apoE−/− mice aggravates disease in this study may appear puzzling. However, the apoE−/− scid/scid mice lack both B and T cells. Therefore, any effects of B cells, including antibody-producing plasma cells derived from them as well as effects of CD8+ T cells, were absent in the present transfer study. In addition, the antigen-presenting cells in apoE−/− scid/scid are never directly exposed to the antigens in the preparation used for immunization, including adjuvant, precluding a possible development of tolerogenic antigen-presenting cells. Furthermore, any early protective mechanisms induced by the immunization may not be transferred if the transferred cells are isolated as late as 20 days after the immunization. The fact that the immunization-induced atheroprotection remains in the mice lacking CD4 suggests that other cells than CD4+ T cells may play an atheroprotective role after immunization. Recent reports also suggest that humoral immune responses may have protective effects on disease development. Because immunization with oxLDL enhances both cellular and humoral immune responses to the atherosclerosis-related antigen, it is possible that some of the mechanisms activated by immunization are deleterious, whereas others are protective. The timing for the induction of such reaction may also be relevant. Further studies are necessary to explore how the balance between such protection and aggravation induced by immunization can be controlled.

To summarize, the present data identify by adoptive transfer methodology that oxidatively modified LDL is an autoantigen that promotes atherosclerosis in hypercholesterolemic mice.
References

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Figure I

A

IgG anti-KLH

- Unimmunized
- KLH
- MDA-LDL

serum dilution: 1/100  1/1000  1/10000

Optical density

B

IgG anti-MDA-LDL

- Unimmunized
- KLH
- MDA-LDL

Optical density

serum dilution: 1/100  1/1000  1/10000
Figure I. Induction of T cell dependent IgG antibody responses in immunized donor mice. ELISA of sera from donor B6 mice immunized with KLH (A) and MDA-LDL (B) show induction of high-titer IgG antibodies to the specific immunogen but not to another antigen. n=6.