Involvement of the CD1d–Natural Killer T Cell Pathway in Neointima Formation After Vascular Injury

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Abstract—Recent studies have established that the immune system plays an important role in the development of atherosclerosis. However, its role in regulating the arterial response to mechanical injury is less well studied. Arterial injury is associated with local accumulation of antibodies, and mice lacking functional T and B cells exhibit increased neointima formation, indicating that adaptive immune responses to neoantigens in the damaged tissue modulate the vascular repair process. To study the role of lipid antigen presentation in the arterial response to injury, we analyzed neointima formation in mice deficient in the lipid antigen-presenting molecule CD1d using a carotid collar model. As compared with control mice, neointima formation was reduced by >60% (P<0.01) in CD1d−/− mice. Moreover, carotid injury of wild-type C57BL/6 mice was associated with expansion of CD1d-restricted natural killer T cells in the spleen and accumulation of natural killer T cells in the periadventitial space of injured arteries. The results suggest that presentation of lipid antigens through the CD1d–natural killer T cell pathway modulates vascular repair responses. (Circ Res. 2007;101:e83-e89.)

Key Words: arterial injury ■ antigen presentation ■ neointima

Mechanisms involved in the vascular response to injury play important roles in atherosclerosis and in-stent restenosis.1,2 Oxidized low-density lipoprotein (LDL) represents a major cause of injury in atherosclerosis and interacts directly and indirectly with components of the innate immune system to induce an inflammatory response, including expression of adhesion molecules, chemokines, and recruitment of monocytes and T cells to the arterial wall.3,4 More recently, it also has become evident that adaptive immune responses to oxidized LDL both contribute to and modulate the disease process. Antigen-presenting cells, such as dendritic cells and macrophages, present lipid and peptide antigens derived from oxidized LDL on surface CD1 and major histocompatibility complex class II molecules, respectively, to activate specific proinflammatory T cells.1 Inhibition of each of these antigen-presenting pathways results in reduced atherosclerosis.5–10 Recent studies also suggest that modulation of autoimmune responses against epitopes in oxidized LDL represents a novel target for prevention and treatment of cardiovascular disease.11,12

The role of the immune system in the vascular response to mechanical injury is less clear. The clinical relevance of this process today is primarily related to the development of in-stent restenosis, a phenomenon that occurs in 22% to 32% of patients treated with bare-metal coronary stents.2 Autopsy studies of human coronary arteries containing stents in combination with experimental studies of the vascular response to injury suggest that stent implantation causes an acute inflammatory reaction mediated by innate immune responses followed by a fibroproliferative repair phase.13,14

Studies in mice have shown that arterial injury is associated with generation of autoantibodies specifically binding to injured but not uninjured arterial tissue, suggesting that adaptive immune responses also contribute to the regulation of vascular repair.15 Further evidence supporting a role of adaptive immunity in the regulation of vascular repair has evolved from studies demonstrating that mice lacking mature T and B cells have enhanced neointima formation after mechanical injury.15–17 To investigate the role of adaptive immunity to lipid antigens in the vascular response to mechanical injury, we used a carotid collar model18 to study neointima formation in CD1d-deficient mice. CD1 molecules are a family of major histocompatibility complex–related molecules expressed by dendritic cells, macrophages, and spleen marginal zone B cells that present lipid and glycolipid antigens to T cells.19 Mice express only CD1d, the only member of this family that interacts with natural killer (NK) T cells.

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Materials and Methods

Animals
CD1d-deficient mice were kindly provided by Chyung-Ru Wang (University of Chicago, Ill). The study was approved by the local ethical committee and conformed to the Guide and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). The animals were fed a regular diet and given water ad libitum.

Periadventitial Collar Injury
At the age of 21 weeks, female CD1d+/−, CD1d−/−, and wild-type C57BL/6 mice were anesthetized with tribromoethanol (Avertin) (0.016 mL/g of 2.5% solution IP), and the right carotid artery was carefully isolated under a dissecting microscope. A nonocclusive plastic collar was placed around the right carotid artery, and the skin incision was closed, as described previously.18 Mice were killed at 3 days after collar placement, and the carotid arteries were perfusion fixed with Histochoice (Amresco). Arteries were dissected out and stored in Histochoice at 4°C until analysis. α-GalCer−treated (Kirin Brewery Company) mice received 0.1 mg of primary antibody served as specificity control. Immunohistochemistry

Morphometric Measurements
The carotid arteries were dehydrated and embedded in paraffin, and 5-µm thick sections were obtained. From the injured carotid artery sections, were collected every 100 µm from the distal end of the segment (4 sections per mouse). Sections were stained with Accustain elastic stain (Sigma), and areas of the different regions and circumferences were calculated using the image software Zeiss Axiovision (Amresco). Arteries were dissected out and stored in Histochoice at 4°C until analysis. α-GalCer−treated (Kirin Brewery Company) mice received 0.1 µg/kg body weight of the drug by IP injection twice a week starting the day before injury. Plasma concentrations of interferon-γ were analyzed by ELISA (Biologend) according to the instructions of the manufacturer.

Flow Cytometry
Deep cervical lymph nodes and spleens were collected from female wild-type, CD1d+/-, and CD1d−/− mice 3 days after collar injury. Cell suspensions were prepared by standard procedures, blocked with 2.4G2 monoclonal antibody, subsequently stained with fluorochrome-conjugated antibodies, and analyzed with a FACS flow cytometer (Becton-Dickinson). The antibodies used in these experiments were fluoroscence isothiocyanate−anti−CD69, phycoerythrin−anti−B220, phycoerythrin−anti−NK1.1, PerCP−anti−CD4, Cy5−anti−CD8α, and antigen-presenting cell−anti−T-cell receptor β (all from BD).

Immunohistochemistry
For IgM and IgG detection, paraffin-embedded carotid arteries were sectioned (5 µm), rehydrated, and subsequently fixed in ice-cold acetone for 5 minutes, washed in PBS twice for 2 minutes, and then permeabilized with 0.5% Triton X-100 in PBS. Sections were washed again twice with PBS for 2 minutes and then blocked with 10% mouse serum in PBS for 30 minutes. The slides were then rinsed in PBS and incubated with biotinylated anti-mouse IgM or IgG antibodies (Vector Laboratories) for 50 minutes for detection of mouse autoantibodies. Smooth muscle cells were identified using a monoclonal anti-mouse smooth muscle α actin antibody (Sigma) and macrophages with a rat anti-mouse Mac-2 (Cedar Lanes Laboratories) with appropriate secondary antibodies. The reaction products were visualized with Vectastain ABC elite kit (Vector laboratories) using diaminobenzidine as substrate (Vector laboratories). Omission of primary antibody served as specificity control.

Confocal Immunofluorescence
Immunofluorescence experiments were performed and analyzed as described previously. Briefly, paraffin-embedded carotid arteries were sectioned (5 mm), rehydrated, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Sections were then blocked for 2 hours with 2% BSA in PBS. Primary antibodies, anti-mouse CD1d (BioSite; biotin conjugated) and MOMA-2 (Biomedicals AG, Augst, Switzerland), were applied overnight at 4°C. The fluorescent nucleic acid dye SYTOX Green (1:300; Molecular Probes) was used for nuclear identification. After washing, the vessels were mounted (Aqua Polymount mounting medium; Polysciences) and examined at ×10 and ×63 magnification (numerical aperture, 0.3 and 1.4, respectively) in a Zeiss LSM 5 Pascal laser-scanning confocal microscope. CD1d and MOMA-2 expressions were detected by monitoring phycoerythrin fluorescence (excitation, 545 nm; emission, 575 nm) and Cy5 (excitation, 650 nm; emission, 670 nm; Jackson ImmunoResearch), and nuclei were identified by monitoring green fluorescence (excitation, 503 nm; emission, 524 nm). Specificity of immune staining was confirmed by the absence of fluorescence in arteries incubated with primary or secondary antibodies alone and in sections from CD1−/− mice.

Statistics
The statistical significance between groups was determined by Mann–Whitney test. *P<0.05 was considered significant.

Results

Effect of CD1d Deficiency on Neointima Formation in Response to Collar Injury
To study the role of CD1d-dependent lipid antigen presenta tion in regulation of neointima formation, we performed collar injury on CD1d−/− mice using heterozygous litter mates as controls. Neointima formation was found to be reduced by more than 60% in CD1d−/− mice as compared with control mice (Figures 1 and 2). CD1d−/− mice also had a significantly lower medial area (35.7 ± 9.1 versus 41.3 ± 6.4×103 mm2; *P<0.05) and intima/media ratio than the control mice (0.094 ± 0.076 versus 0.21 ± 0.15; *P<0.05). There were no major morphological differences between uninjured carotid arteries from CD1d−/− and CD1d+/− mice (Figure 2). Small areas of macrophage infiltration could be observed in both the neointima and
media of injured arteries, but there was no significant difference between CD1d−/− mice and control mice in this respect (19.6±14.1% versus 10.9±12.5% and 8.3±7.5% versus 9.1±8.2% of all cells, respectively; Figure 3). There was also no difference in neointimal α-actin staining between CD1d−/− and control mice (Figure 3). Cells expressing CD1d were found in the periadventitial area of injured control mice (Figure 4). Parallel staining with for CD1d and macrophages demonstrated that only ≈25% of the macrophages expressed CD1d (Figure 5). To investigate whether the role of CD1d in regulating the vascular response to injury involved a general activation of NK T (NKT) cells, C57BL/6 wild-type mice were treated with α-galactosylceramide (α-GalCer), a synthetic antigen that activates NKT cells through binding to CD1d.21 However, treatment with α-GalCer did not influence the response to collar injury, as assessed by measurement of carotid neointima formation after 3 weeks (4.3±3.3×10−3 versus 5.9±4.8×10−3 mm² in control mice; P=NS). There were no detectable levels of interferon-γ in plasma of injured
Deposition of Antibodies in Injured Arteries

Activation of antibody expression against lipid and peptide antigens on damaged or apoptotic cells represents a possible mechanism through which adaptive immunity may regulate the vascular response to injury. Immunohistochemical analysis of antibody deposition in the carotid artery of C57BL/6 wild-type mice 3 weeks after injury demonstrated the presence of IgG in the adventitia and in the neointima but not in the media (Figure 6). A similar pattern of IgG deposition was observed in CD1d<sup>−/−</sup> mice (Figure 6). IgM was present in the adventitia, media, and neointima in all animals (Figure 6).

Effect of Collar Injury on Draining Lymph Node and Spleen Immune Cells

The effect of injury on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, NKT cells, NK cells, and B cells in the spleen and draining (cervical)

Figure 4. Confocal immunofluorescence images showing CD1d staining (red) in injured carotid arteries from wild-type control (B) and CD1d<sup>−/−</sup> (D) mice. Sections were costained with the DNA-binding dye SYTOX Green (green; A and C for wild-type control and CD1d<sup>−/−</sup>, respectively). The white arrow indicates an area rich in CD1d-positive cells. Bars=200 μm. E, Higher-magnification image from an injured artery, as in A and B, showing CD1d-positive cells (white arrows) in the adventitial layer. Bar=10 μm.

CD1d<sup>−/−</sup>, CD1d<sup>+/−</sup>, or C57BL/6 wild-type mice treated with α-GalCer at the 3-week time point.
lymph nodes was studied by flow cytometry at day 3 after the collar implantation in C57BL/6 wild-type mice. Expression of CD69 was used as a marker of leukocyte activation. Injury was associated with a 28% increase in the spleen NKT cell population (1.14±0.23% of lymphocytes versus 0.89±0.16% in uninjured control mice; P<0.05; Figure 6), and there was also a trend toward an increased expression of the activation marker CD69 on these cells (66.3±8.8% versus 60.2±2.7% in uninjured control mice; P=0.08). The fraction of CD4+ T cells in the spleen decreased from 20.0±2.2% in control mice to 16.1±2.2% (P<0.005) in injured mice, which was associated with a relative increase in the B-cell fraction from 56.6±4.6% to 60.9±6.2% (Figure 7; P=NS).

Cells isolated from lymph nodes were pooled because of the low number of cells obtained from each animal. Three days after injury, there was no difference in the cellular composition of draining lymph nodes isolated from injured and control arteries with respect to CD4+ T cells (24.8% versus 26.1%), CD8+ T cells (21.7% versus 19.3%), NKT cells (0.7% versus 0.7 1%), NK cells (0.9% versus 1.0%), and B cells (49.5% versus 49.7%). There was also no difference in the expression of the activation marker CD69 between injured and control mice on any of the cell types studied.

The effect of injury on leukocyte populations in CD1d−/− mice was studied in less detail. As expected, CD1d−/− mice had fewer NKT cells in the spleen (0.24±0.07%; n=4) and in draining lymph nodes (0.16% based on lymph nodes pooled from 4 mice). Injured CD1d−/− mice also had fewer NKT cells in the spleen than CD1d−/+ mice (0.47±0.08% versus 0.88±0.13% based on 2 separate experiments on cells pooled from 3 mice), whereas there was no difference in the fraction of CD4+ T cells.

### Discussion

The present study demonstrated (1) that vascular injury is associated with an expansion of CD1d-regulated NKT cells in the spleen, (2) that CD1d-expressing cells are present in the periadventitial area of injured arteries, and (3) that mice deficient in the lipid antigen–presenting molecule CD1d have reduced neoantigen formation in response to injury of the carotid artery. The results suggest that immune responses to lipid antigens modulate vascular repair responses.

Several previous experimental studies have indicated that the primary function of the immune system in vascular injury is to limit the extent of the subsequent repair process. The first indication of an inhibitory effect of the immune system on the vascular repair process was obtained from experiments showing increased neointimal formation on balloon angioplasty in rats lacking functional T cells.16 The inhibitory role of T cells was subsequently supported by studies demonstrating an inhibitory effect of the T cell cytokine interferon-γ on neoantigen formation and smooth muscle cell proliferation,22 as well as by the observation of increased neoantigen formation in collar-injured CD40 ligand–deficient mice.17 Also Rag-1 knockout mice, which lack both mature T and B cells, are characterized by increased neoantigen formation in response to arterial injury.15

Interestingly, the extensive neoantigen formation in Rag-1 knockout mice has been shown to be prevented not only by T-cell transfer but also by B-cell transfer.15 The latter observation suggests that humoral immune responses also have a role in limiting the vascular response to injury, a notion that is supported by the finding of IgM and IgG accumulation at sites of vascular injury.15 Taken together, these studies provide strong support for the concept that T and B cells activated by arterial tissue injury serve to limit the extent of the subsequent vascular repair process. The mechanisms involved in this downregulation of vascular repair processes remain to be fully understood but may include inhibition of smooth muscle cell growth by T cell–derived interferon-γ and antibody-mediated removal of proinflammatory debris. It also remains to be elucidated how these immune responses are activated. One possible mechanism could be that injury is associated with formation of neoantigens in damaged cells and extracellular tissue and that presentation of these neoantigens by macrophages and den-
dritic cells activates an adaptive immune response that serves to clear these structures and modulate the subsequent repair process. In the present study, we tested the hypothesis that this is mediated by a CD1d-dependent presentation of lipid antigens. Our results clearly demonstrate that the inhibitory role of adaptive immunity in injury-induced neointima formation does not involve immune responses to lipid antigens. In contrast, the present findings demonstrate the existence of an adaptive immune response that promotes neointima and suggest that presentation of lipid antigens by CD1d for NKT cells may be required for a normal vascular repair process to occur. The functional role of NKT cells remains to be fully understood, but experimental studies suggest that they are involved in protection against infection, tumors, and autoimmunity. Most NKT express an invariant Vα14-Jα18 T-cell receptor α-chain and respond with production of both T helper (Th)1 and Th2 cytokines when activated. It has also been demonstrated that NKT cells mediate injury-induced immunosuppression possibly to limit autoimmune responses against lipids modified by cellular and tissue damage. In the vasculature, on the other hand, LDL receptor–null and apolipoprotein E–null mice that lack CD1d develop less normal development of the artery wall.

Immunosuppression possibly to limit autoimmune responses against lipids modified by cellular and tissue damage. In the vasculature, on the other hand, LDL receptor–null and apolipoprotein E–null mice that lack CD1d develop less normal development of the artery wall. However, although no major morphological differences were observed between uninjured CD1d−/− and CD1d+/− arteries, it cannot be excluded that the absence of CD1d−/− affects the normal development of the artery wall.

Administration of the synthetic glycolipid α-GalCer induces a rapid activation of NKT cells, resulting in production of both Th1 and Th2 cytokines that subsequently activate other immune cells, including NK cells, dendritic cells, and T and B cells. Treatment with α-GalCer has been shown to prevent autoimmunity in animal models of type 1 diabetes and experimental autoimmune encephalomyelitis but increases early lesion formation in hypercholesterolemic mice. The reasons for the lack of effect of α-GalCer on neointima formation in response to vascular injury remains to be fully understood but implies that activation of NKT cells is required for the normal repair processes to occur but does not have the capacity to promote fibroproliferative responses beyond that.

Vascular repair responses to mechanical injury are considered to play a key role in the development of in-stent restenosis. The incidence of in-stent restenosis has been significantly reduced by the introduction of drug-eluting stents coated with substances such as rapamycin (sirolimus) and paclitaxel. Both drugs are immunosuppressive, but their effect on in-stent restenosis has been attributed primarily to an antiproliferative effect on smooth muscle cells. However, recent concerns that the use of drug-eluting stents may be associated with increased long-term cardiovascular mortality resulting from disturbed vascular healing and late in-stent thrombosis has made it important to reach a better understanding of the underlying biological processes. The present results demonstrate that in addition to the previously described inhibitory effects of adaptive immunity on neointima formation, stimulatory immune responses also exist, and that presentation of lipid antigens through the CD1d-NKT cell pathway may be required for normal vascular repair to occur. Taken together, these observations suggest that the immune system remains a possible target for local intervention against in-stent restenosis and points to the need for a better understanding of the mechanism of action of stents coated with immunomodulatory drugs.

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Disclosures

None.

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